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A METHOD FOR DETECTING SMALL QUANTITIES OF CHLORETONE (TRICHLOROTERTIARYBUTYL ALCOHOL) IN AQUEOUS SOLUTIONS.

By T. B. ALDRICH.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

CORRECTION.

In Volume XXXIII, No. 3, March, 1918, page 528, line 12, for *Hoffmann* read *Hodgkin*.

On page 528, heading to Table II, and page 529, line 1, for *hoffmanni* read *hodgkini*.

a partly filled bottle or container, the compound will be found partly sublimed on the upper walls of the vessel in the form of beautiful needle-shaped crystals.

Although only qualitative results are claimed for the method, still by using duplicate apparatus, especially where small amounts (1 to 5 mg.) are present, the amount may be estimated quite closely. The entire apparatus necessary consists of two flasks, holding 1 liter and 300 cc., respectively, a condenser, and a steam generator. The larger flask is used for the steam distillation, while the smaller is used for the return flow condenser. With this apparatus, one can detect very readily in 300 to 500 cc. of water

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By T. B. ALDRICH.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

(Received for publication, March 7, 1918.)

The wide use of chloretone therapeutically, especially at the present time, as a hypnotic, an anesthetic, a sedative, etc., seems to call for the publication of the following method for its detection.

Part of the work in connection with the development of this method was carried out several years ago, while quite recently this older work has been repeated and the observations made at that time reconfirmed. The efficacy of the method under varying conditions, also its limitations to a certain extent, has been studied.

The method is based on the volatility of chloretone, one of its chief physical properties, its insolubility in water, and also on its property of crystallizing in needles from aqueous solutions on cooling. All who are familiar with chloretone have been impressed with its volatility in the air, or with steam and water. The substance cannot be dried without losing in a very short time considerably in weight; nor can it be kept in an open vessel or even wrapped in ordinary paper any length of time. If kept in a partly filled bottle or container, the compound will be found partly sublimed on the upper walls of the vessel in the form of beautiful needle-shaped crystals.

Although only qualitative results are claimed for the method, still by using duplicate apparatus, especially where small amounts (1 to 5 mg.) are present, the amount may be estimated quite closely. The entire apparatus necessary consists of two flasks, holding 1 liter and 300 cc., respectively, a condenser, and a steam generator. The larger flask is used for the steam distillation, while the smaller is used for the return flow condenser. With this apparatus, one can detect very readily in 300 to 500 cc. of water

1 to 5 mg. of chloretone, and with a constricted tube cooled with air instead of a condenser, as little as 0.5 mg. or less. In my earlier experiments a constricted air-cooled tube was employed, while later I employed a condenser exclusively.

When a large amount of chloretone is present, it may be observed in the condenser during the steam distillation; but where only a few mg. or a fraction of a mg. are present, the use of the return flow condenser or tube is absolutely necessary for its recognition. The crystals of chloretone in the latter case are noticed usually at the lower cooled portion of the condenser, especially where the steam crest is being condensed, or possibly a few crystals may be observed according to quantity present, about one-third or at the most half way up the condenser. Where a liquid is employed, it is only necessary to pass steam through it; when a solid contains chloretone this should be powdered and then introduced into the steam distillation flask with the water.

Not only may chloretone be detected with certainty in aqueous solution when alone, but it may also be recognized when mixed with other bodies with which it may be confused, or which may interfere to a certain extent with its detection.

In the presence of volatile organic solvents as alcohol, acetone, etc., it is practically impossible to detect small amounts of chloretone, since the solvent keeps the drug in solution and prevents crystallization. It may be detected with a considerable degree of certainty, however, in a mixture of oil, protein, fat, salt, etc., which does not contain the volatile solvents mentioned above or other volatile bodies with which it may be confused.

Chloretone is not apt to be confused with other volatile compounds of similar use, but, if present to any great extent, may be identified and distinguished from these bodies by further specific tests. The analogous body, brometone, although volatilizing, etc., similar to chloretone, forms, when distilled in small amounts, small crystals instead of needles as in the case of chloretone. Camphor, which also volatilizes with steam, does not give needles as in the case of chloretone, nor does it give the flame test with copper oxide.

Chloral hydrate and chloretone may be confused with each other, but here the greater solubility of chloral hydrate and the comparative insolubility of chloretone in water, together with its odor, will serve to differentiate these bodies.

In the presence of coagulable protein, I have been unable to detect very small amounts of chloretone, but after digesting the protein with pepsin HCl (thus preventing coagulation) as small amounts may be detected as in water alone.[1]

Oils or fats do not in general interfere with the detection of small amounts of chloretone.

EXPERIMENTAL.

A. Detection of Chloretone in Aqueous Solution.—5 gm. of chloretone were dissolved in 1,000 cc. of H_2O, and portions of this solution used for the following tests:

(1) To 1 cc. of the chloretone solution (5 mg.), placed in a steam distillation flask, were added about 300 cc. of H_2O. It was distilled with steam until 100 cc. of the distillate had been collected.

This 100 cc. of distillate containing the 5 mg. of chloretone was transferred to a small flask and boiled for half an hour, using a return flow condenser. Crystals of chloretone were noticed in considerable quantity in the condenser. From the amount present in this experiment, it was estimated that as little as 0.25 mg. or even less might be detected.

(2) The above was repeated but with only 0.5 cc. of the chloretone solution (2.5 mg.). *Positive.*

(3) Repeated (2). *Positive.*

(4) Repeated the experiment using 0.25 cc. of the chloretone solution (1.25 mg.). *Positive, but slight amount.*

(5) Repeated (4). *Positive.*

(6) Repeated the experiment using 0.05 cc. of the chloretone solution (0.25 mg.). *Negative.*

B. Detection of Chloretone When Dissolved in Horse Serum.—(1) 1 cc. of chloretone solution, 0.5 per cent (5 mg.), was added to 250 cc. of normal horse serum and the test carried out in the usual way. *Negative.* No doubt the coagulation of the serum interferred with the distillation of the chloretone, especially in this small amount.

(2, *a*) 120 cc. of serum containing 5 mg. of chloretone were digested at 37–39° C. with pepsin hydrochloric acid for 4 days. *Positive. Very pronounced.*

(*b*) (*a*) was repeated except that only 1 cc. of chloretone solution (1 mg.) was employed. *Positive. Slight trace.*

C. Detection of Chloretone in the Presence of Water and Oil.—(1) 2 cc. of chloretone solution (10 mg.) were placed in a flask, 200 cc. of distilled water and 0.5 cc. of olive oil added and distilled in the usual way. *Positive.*

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[1] In a paper to be published shortly this feature of digestion is used in detecting chloretone in animal fluids and organs.

(2) 1 gm. of chloretone dissolved in olive oil (5 cc.) was placed in a flask, 200 cc. of water were added, and distilled. *Positive.*

D. Detection of Chloretone When Mixed with Various Substances.—The following substances were placed in a steam distillation flask:

0.5	cc.	propionic acid.
0.5	"	butyric "
0.2	gm.	stearic "
0.2	"	palmitic "
1.0	"	dextrose.
0.5	"	dried horse serum.

To the above mixture 1 cc. of chloretone solution 0.5 per cent was added (5 mg.) and then 150 cc. of water. This mixture was subjected to steam distillation. Chloretone *positively recognized.*

E. Detection of Chloretone in Alcoholic Solution of Various Strengths.—(1) 1 cc. of an aqueous 0.5 per cent solution of chloretone (5 mg.) was placed in a flask, 100 cc. of 10 per cent alcohol were added, and distilled with steam. *Negative.*

(2) Repeated (1) except that 100 cc. of 5 per cent alcohol were used. *Negative.*

(3) Repeated (1) except that 100 cc. of 1 per cent alcohol were employed. *Negative.*

From experiments carried out with organic solvents (alcohol) it is shown that chloretone, especially in small amount, 1 to 5 mg., cannot be detected by this method even in the presence of a very small amount of alcohol. As far as investigated, even small amounts of volatile organic solvents dissolve the small amount of chloretone present, thus preventing its recognition.

F. Detection of Chloretone When Mixed in Small Quantities with Other Volatile Compounds.—The following products were placed in a steam distillation flask, about 300 cc. of water added and distilled until about 100 cc. of distillate had passed over.

(1) 5 mg. brometone crystals.

(2) 1 cc. chloretone inhalent.

(3) 1 gm. camphor.

(4) 15 mg. camphor, 15 mg. chloretone.

(5) 0.2 gm. chloral hydrate.

(6) 15 mg. chloral hydrate, 15 mg. of chloretone.

(1) Did not give typical needles as by chloretone.

(2) Semicrystalline sublimation obtained. Chloretone may be detected in this product by means of the copper-flame test (green).

(3) Did not give typical needles (applied copper-flame test). *Negative.*

(4) Did not give typical needles (applied copper-flame test). *Positive.*

(5) Did not give typical crystals—solubility of portion sublimed in water distinguished from chloretone.

(6) Did not give very pronounced needles; however, some were present. Was separated by water.

The method lends itself especially to the detection of chloretone in toxicological and pharmacological work. Here we have the drug usually unassociated with bodies that would interfere with its recognition, and a very small amount may be detected.

SUMMARY.

Very small quantities of chloretone may be detected with certainty in aqueous solutions by distilling with steam and boiling the distillate on a reflux condenser.

The method employed is based on the physical properties of chloretone: volatility with steam, insolubility in water, property of crystallizing in the form of needles.

Chloretone may be detected when accompanied by other substances such as oils, fats, acids, salts, etc. The organic solvents, however, prevent its recognition.

In the presence of substances which simulate chloretone, specific tests may be made to differentiate them.

The method lends itself admirably to the detection of the drug in the fluids and tissues of the body.

THE ADRENALS IN RELATION TO CARBOHYDRATE METABOLISM. I.

THE INFLUENCE OF REPETITION OF EPINEPHRINE INJECTION UPON THE INTENSITY OF GLYCOSURIA AND HYPERGLYCEMIA AND THE GLYCOGEN CONTENT OF THE LIVER.

By SHIGENOBU KURIYAMA.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, March 6, 1918.)

Epinephrine glycosuria, first discovered by Blum,[1-2] results from hyperglycemia.[3-4] Doyon and Kareff,[5] Drummond and Paton,[6] Bierry and Gatin-Gruzewska,[7] Wolownik,[8] and Agadschanianz[9] demonstrated a marked loss or complete disappearance of the liver glycogen after epinephrine injection. According to Gatin-Gruzewska,[10] the liver and muscles of rabbits, previously fasted for 24 hours, were glycogen-free in 36 to 40 hours after intraperitoneal injection of epinephrine in doses of 1 mg. or less per kilo of body weight. The complete disappearance of the muscle glycogen in rabbits, fasted and poisoned with epinephrine, was also reported by Agadschanianz.[9] Paton,[11] after epinephrine injection, obtained a more marked glycosuria in well fed animals than in those which had been taking less food. Contrary to many reports concerning the influence of epinephrine in causing liver glycogen loss, Loeper and Crouzon[12] reported that they found an increased glycogen content of the liver after an intravenous

[1] Blum, F., *Deutsch. Arch. klin. Med.*, 1901, lxxi, 146.

[2] Blum, *Arch. ges. Physiol.*, 1902, xc, 617.

[3] Zuelzer, G., *Berl. klin. Woch.*, 1901, xxxviii, 1209.

[4] Metzger, L., *Münch. med. Woch.*, 1902, xlix, 478.

[5] Doyon, M., and Kareff, N., *Compt. rend. Soc. biol.*, 1904, lvi, 66.

[6] Drummond, W. B., and Paton, D. N., *J. Physiol.*, 1904, xxxi, 92.

[7] Bierry, H., and Gatin-Gruzewska, Z., *Compt. rend. Soc. biol.*, 1905, lviii, 902.

[8] Wolownik, B., *Virchows Arch. path. Anat.*, 1905, clxxx, 225.

[9] Agadschanianz, K., *Biochem. Z.*, 1907, ii, 148.

[10] Gatin-Gruzewska, Z., *Comp. rend. Acad.*, 1906, cxlii, 1165.

[11] Paton, D. N., *J. Physiol.*, 1903, xxix, 286.

[12] Loeper, M., and Crouzon, O., *Comp. rend. Soc. biol.*, 1903, lv, 1376.

injection of epinephrine. Pollak[13] made rabbits glycogen-free by fasting and strychnine poisoning, and then by small increasing doses of epinephrine was able to bring about a new formation of the liver glycogen, in such quantities as can be equaled only in animals fed on carbohydrate.

It has often been reported that epinephrine does not produce glycosuria in animals which have been made entirely or almost glycogen-free. Blum[1] at first supposed that epinephrine glycosuria occurs in animals, fasted entirely glycogen-free; but later he found it necessary to modify his opinion.[2] Herter and Richards[14] reported that prolonged fasting with phlorhizin poisoning reduced a dog to a condition in which administration of epinephrine resulted in little or no glycosuria, phlorhizin, however, still being able to cause glycosuria. Ringer[15] made dogs glycogen-free by a combination of hunger, cold, and phlorhizin, and then found that epinephrine injection induced no increase in the elimination of sugar, nor any change in the dextrose-nitrogen ratio. Frank and Isaac[16] and Michaud[17] failed to demonstrate epinephrine hyperglycemia in animals poisoned with phosphorus. Doyon and Kareff,[5] Wolownik[8] and many others consider that the liver glycogen is, for the most part, the source of the sugar eliminated by administration of epinephrine. Falta and Priestley,[18] using rabbits, tied off the liver vessels and found that despite a subcutaneous injection of epinephrine, the blood sugar content did not increase as in the controls. Michaud[17] reported that in dogs with an Eck fistula, epinephrine did not cause any rise in the blood sugar. In frog experiments, Velich[19] found that the spleen, intestine, reproductive glands, or pancreas may be removed without any effect upon epinephrine glycosuria, but removal of the liver prevents glycosuria. Starkenstein[20] found that when normal livers are perfused with salt solution or defibrinated blood, the addition of epinephrine to the perfusion fluid has no influence upon the rate of disappearance of glycogen. He attributes the negative results to the early death of the peripheral nervous mechanism in the excised liver. Macleod and Pearce[21] proved that injection of epinephrine into the portal circulation of a living animal is followed by increased glycogenolysis in the liver and considered this to be due to the local glycogenolytic mechanism. Bang[22] noticed that epinephrine hyperglycemia in fasted rabbits is as high as in well nourished controls; in the former, however, it appeared a little later

[13] Pollak, L., *Arch. exp. Path. u. Pharm.*, 1909, lxi, 149.

[14] Herter, C. A., and Richards, A. N., *Med. News*, 1902, lxxx, 201.

[15] Ringer, A. I., *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 8.

[16] Frank, E., and Isaac, S., *Z. exp. Path. u. Therap.*, 1910, vii, 326.

[17] Michaud, *Verhandl. deutsch. Kong. inn. Med.*, 1911, xxviii, 561.

[18] Falta, W., and Priestley, J. G., *Berl. klin. Woch.*, 1911, xlviii, 2102.

[19] Velich, A., *Virchows Arch. path. Anat.*, 1906, clxxxiv, 345.

[20] Starkenstein, E., *Z. exp. Path. u. Therap.*, 1912, x, 78.

[21] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1911–12, xxix, 419.

[22] Bang, I., Der Blutzucker, Wiesbaden, 1913, 89.

than in the latter. He considers, therefore, that the liver is indispensable for epinephrine hyperglycemia, but the liver glycogen, on the other hand, is not a necessary factor.

Wilenko[23] reported that epinephrine depresses the physiological elevation of the respiratory quotient after administration of carbohydrate, and that glucose administered intravenously into animals taking epinephrine appears quantitatively in the urine. He concluded that epinephrine diminishes the ability of the organism to burn sugar. La Franka[24] found no change of the respiratory quotient after epinephrine administration. Hári,[25] Fuchs and Róth,[26] Bernstein and Falta,[27] and Lusk[28] found an increase of the respiratory quotient after epinephrine injection in normal men, and dogs and patients with Addison's disease.

Blum[2] observed that in fasted dogs, in which epinephrine was insufficient to cause dextrose elimination, a diet of olive oil brought it about. Eppinger, Falta, and Rudinger[29] claim that fat metabolism is increased in epinephrine glycosuria and that fat feeding increases the sugar excretion. Though some authors reported that epinephrine has no influence upon protein metabolism (Blum,[2] Wolownik,[8] Kraus and Friedenthal[30]) Eppinger, Falta, and Rudinger [29] and Quest[31] noticed an increased nitrogen elimination after epinephrine injection. Paton[11] reported that epinephrine does not change the protein metabolism of well nourished animals. In insufficiently nourished animals, however, he observed a distinct increase in the nitrogen excretion. This was also confirmed by Underhill and Closson.[32]

As to the influence of repetition of epinephrine injection upon the glycosuria, Herter and Wakeman[33] reported that the glycosuric effect of epinephrine disappears in a very few days. Injecting epinephrine into rabbits for 11 to 25 days, Paton[11] noticed that the animals could be made to withstand an otherwise lethal dose of epinephrine. Glycosuria was usually present. Velich[13] observed that in frogs glycosuria was always induced by daily repetition of epinephrine injection as long as a good nutritional condition was kept. When the animals were fasted from the start of the experiments, after 5 to 6 days, glycosuria could no longer be induced.

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[23] Wilenko, G. G., *Biochem. Z.*, 1912, xlii, 44.

[24] La Franka, S., *Z. exp. Path. u. Therap.*, 1909, vi, 1.

[25] Hári, P., *Biochem. Z.*, 1911, xxxviii, 23.

[26] Fuchs, D., and Róth, N., *Z. exp. Path. u. Therap.* 1911–12, x, 187.

[27] Bernstein and Falta, W., *Verhandl. deutsch. Kong. inn. Med.*, 1912, xxix, 536.

[28] Lusk, G., *Arch. Int. Med.*, 1914, xiii, 673.

[29] Eppinger, H., Falta, W., and Rudinger, C., *Z. klin. Med.*, 1908, lxvi, 1.

[30] Kraus, F., and Friedenthal, H., *Berl. klin. Woch.*, 1908, xlv, 1709.

[31] Quest, R., *Z. exp. Path. u. Therap.*, 1908, v, 43; cited by Allen, F. M., Studies Concerning Glycosuria and Diabetes, Boston, 1913, 690.

[32] Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1906–07, xvii, 42.

[33] Herter, C. A., and Wakeman, A. J., *Virchows Arch. path. Anat.*, 1902, clxix, 479.

He considers that this was due to the entire loss of the liver glycogen. When Pollak injected epinephrine repeatedly into rabbits which were fasted from the beginning of the experiments, glycosuria usually disappeared in several days, while the blood sugar content still reached as high as 0.29 to 0.4 per cent. His explanation was that the kidneys became accustomed to a high blood sugar content or they received some specific influence by repeated epinephrine injection.

From the brief references mentioned above, it will be seen that there is still a great uncertainty concerning the mechanism of epinephrine hyperglycemia and glycosuria. My present experiments were chiefly planned to see how the intensity of epinephrine hyperglycemia and glycosuria is modified by repetition of epinephrine injection or the nutritional condition of the animals, and also to see how the liver glycogen is affected by epinephrine injection.

Methods.

Rabbits were employed. They were divided into three groups. In the first, epinephrine was injected daily, the animals being kept in a good nutritional condition. After a considerable length of time, the animals were fasted, the epinephrine injection being continued. The urinary sugar was estimated daily; the blood sugar from time to time. The rabbits were then killed and the glycogen content of the liver was determined. In the second group, the animals were fasted from the beginning of the experiment. Daily epinephrine injection, the determination of the urine and blood sugar and of the liver glycogen were the same as in the first group. The glycogen of the skeletal muscles was also estimated. In two rabbits of this group, instead of daily epinephrine injection, this drug was injected only once, at the end of the fasting period. The third group was used as control, the glycogen content of the liver and the muscles being estimated after a certain period of fasting. The preparation of epinephrine employed was adrenalin (1 : 1,000) of Parke, Davis and Company. It was injected subcutaneously without any dilution. In the preliminary period all the rabbits were well fed on oats and vegetables, and at the start of the experiments the animals selected were as nearly as possible in like nutritional condition. In the feeding period of the first group, the same food was continued. Water was always given freely to all the animals. The urine was collected daily by pressure upon the bladder through the abdom-

inal wall. The urine was examined for protein and casts from time to time.

The sugar in the urine was determined by the Pavy-Kumagawa-Suto method.[34] As the original apparatus, which prevents reoxidation of the copper solution was not obtainable, a similar device was made with the valve of a rubber bulb. The valve, which was connected to the flask containing the copper solution by a rubber tube, was placed in water in a flask. The ammonia escaping from this flask was received in another flask containing diluted sulfuric acid.

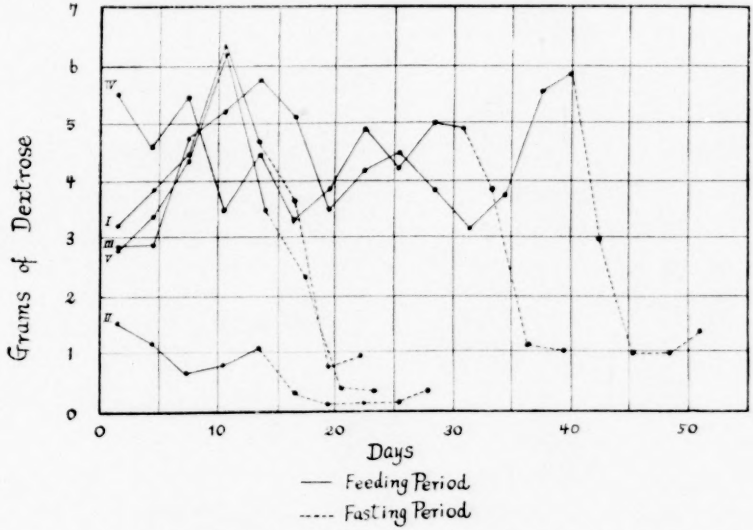

CHART I. The influence of repetition of epinephrine injection upon the intensity of glycosuria (cf. Table I).

At the end of the experiment, the rabbit was rendered unconscious by a blow on the neck and bled. The glycogen of the liver and skeletal muscles (hind legs) was determined by Pflüger's method. The glycogen was hydrolyzed by hydrochloric acid, and the sugar thus produced was determined by the Pavy-Kumagawa-Suto method. The sugar content of the blood was determined by the Lewis-Benedict method. The results are shown in Tables I, II, and III and Chart I. In making Chart I, the

[34] Kinoshita, T., *Biochem. Z.*, 1908, ix, 208. Neubauer, C., and Vogel, J., Analyse des Harns, Wiesbaden, 11th edition, 1910, 399.

TABLE I.

Influence of Repetition of Epinephrine Injection upon the Intensity of Glycosuria and Hyperglycemia and the Glycogen Content of the Liver (See Chart I).

Rabbits Were First Fed and Then Fasted.

Rabbit	I ♀	*II ♀	III ♀	IV ♀	V ♀
Amount of epinephrine per kilo of initial body weight injected daily, *mg.*	0.75	0.5	1.0	1.0	1.0

Glycosuria.

Experimental day.	Urine.									
	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.
	cc.	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*
1	284	3.11	111	1.54	315	2.59	201	5.29	241	3.19
2	131	4.31	56	1.42	140	2.43	204	5.56	138	2.11
3	120	2.18	53	?	136	3.49	231	5.64	102	3.14
4	133	3.41	76	1.09	140	2.99	184	4.60	213	2.66
5	130	2.69	66	0.97	136	2.48	146	3.44	279	4.16
6	210	5.38	70	1.49	158	3.10	162	5.79	186	3.35
7	227	4.73	80	0.70	142	4.41	172	6.25	144	2.83
8	230	?	98	?	146	4.56	186	5.64	163	4.87
9	167	4.17	41	0.56	158	5.26	183	4.36	243	5.46
10	189	6.52	67	0.74	184	6.70	173	3.43	248	7.19
11	262	6.81	100	0.76	173	3.64	135	2.87	196	6.53
12	377	5.66	76	0.91	208	5.27	139	4.03	142	4.90

13	214	4.87	65	0.86	179	6.63	225	5.49	142	3.95
14	299	4.98	60	0.75	166	5.93	128	4.27	206	3.12
15	184	4.04	96	1.57	133	4.67	323	3.55	174	3.59
16	†310	5.58	†64	0.48	184	5.49	140	4.06	178	3.43
17	282	4.31	58	0.28	152	5.07	142	2.10	†271	4.30
18	127	1.02	50	0.17	146	4.78	199	3.72	110	2.22
19	121	0.66	39	0.14	138	3.33	150	5.00	127	0.56
20	106	0.66	38	0.14	158	3.55	137	3.38	111	0.44
21	115	0.90	45	0.15	220	3.61	171	3.17	102	0.31
22	142	1.20	28	0.12	194	4.13	?	?	137	0.47
23	55	0.74	30	0.15	200	5.88	212	4.1	84	0.65
24			32	0.13	266	2.50	189	5.04	91	0.25
25			27	0.12	227	6.48	161	4.18	78	0.22
26			47	0.22	164	3.53	144	3.65		
27			30	0.13	218	3.36	194	4.73		
28			‡78	0.26	202	4.64	143	4.77		
29			‡92	0.40	152	2.98	244	6.97		
30					170	3.82	128	3.33		
31					124	2.51	130	3.21		
32					111	1.93	204	6.58		
33					197	4.99	†125	4.04		
34					159	3.46	151	4.51		
35					140	2.5	150	2.94		
36					175	5.08	99	1.12		
37					204	6.92	118	1.37		
38					233	5.07	88	0.86		
39					172	4.59	86	1.01		
40					194	5.06	92	1.03		

TABLE I—*Concluded.*

Experimental day.	Urine.									
	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.
	cc.	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*
41					188	6.60	68	1.06		
42					†157	4.03				
43					163	3.71				
44					107	1.11				
45					129	1.04				
46					205	0.92				
47					208	0.89				
48					186	1.03				
49					119	0.99				
50					123	0.86				
51					117	1.10				
52					174	1.57				
Total amount of dextrose in **fasting** period, *gm.*	1.07		2.89		17.25		17.94		9.45	

Body weight.

Experimental day.		Experimental day.		Experimental day.		Experimental day.		Experimental day.	
	kg.		*kg.*		*kg.*		*kg.*		*kg.*
1	2.12	1	1.88	1	2.28	1	2.11	1	2.38
16	1.88	16	1.86	16	2.12	7	1.94	17	2.02
23	1.36	29	1.55	46	2.36	33	1.92	25	1.30
				52	1.74	41	1.40		

Sugar content of blood.

	per cent		per cent		per cent		per cent		per cent
1	(*a*) 0.380 (*b*) 0.305	1	(*a* 0.316 (*b*) 0.230	1	(*a*) 0.290 (*b*) 0.330	6	(*a*) 0.396 (*b*) 0.308	16	(*a*) 0.397 (*b*) 0.363
15	(*a*) 0.432	15	(*a*) 0.346	15	(*a*) 0.424	32	(*a*) 0.328 (*b*) 0.256	25	(*a*) 0.304 (*b*) 0.272
20	(*a*) 0.542	20	(*a*) 0.253	42	(*a*) 0.462 (*b*) 0.298	36	(*a*) 0.292 (*b*) 0.188		
22	(*a*) 0.467	22	(*a*) 0.243	45	(*a*) 0.287 (*b*) 0.302	39	(*a*) 0.291 (*b*) 0.294		
23	(*a*) 0.393 (*b*) 0.285	23	(*a*) 0.248	48	(*a*) 0.355 (*b*) 0.284				
		24	(*a*) 0.217						
		26	(*a*) 0.227						
		27	(*a*) 0.248						
		28	(*a*) 0.264						
		29	(*a*) 0.285 (*b*) 0.245						

Glycogen content of liver.

Weight of liver, *gm*.....	59.2	37.2	43.3	41.8	39.6
Glycogen *gm*.....	1.076	0.630	2.108	1.740	0.596
Glycogen *per cent*.....	1.82	1.69	4.87	4.17	1.51
Interval between the last epinephrine injection and glycogen determination, *hrs*.....	24	7	24	7	8

* In the last half of fasting period, a slight albuminuria and cylinduria. In the other rabbits no sign of kidney injury.

† Beginning of the fasting period.

‡ 150 cc. of water were given by stomach sound 30 min. before epinephrine injection.

(*a*) and (*b*), 2 and 4 hours respectively after epinephrine injection.

TABLE II.

Influence of Repetition of Epinephrine Injection upon the Intensity of Glycosuria and Hyperglycemia and the Glycogen Content of the Liver and Muscles.

The Rabbits Were Fasted from the Beginning of the Experiments. In Rabbits IV and V Epinephrine Was Injected Only Once.

Rabbit		I ♀	II ♀	*III ♀	IV ♀	V ♀
Amount of epinephrine per kilo of initial body weight, *mg.*	Experimental day.					
	1, 2	0.75				
	3, 4, 6	1.0	1.0 daily.	0.75 daily.		
	5	1.5				
	9				0.93	0.59

Glycosuria.

Experimental day.	Urine.									
	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.	Volume.	Dextrose.
	cc.	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*	*cc.*	*gm.*
1	429	2.77	310	1.17	214	3.57				
2	164	1.84	206	0.67	144	0.92				
3	134	1.74	143	0.62	152	0.27				
4	142	1.54	260	0.47	141	0.27				
5	216	2.77	283	0.27	136	0.23				
6	92	1.05	180	0.48	112	0.22				
7	Found dead.		170	0.46	90	0.18				
8			149	0.81	77	0.16				
9			174	0.61	66	0.15	75	1.53	117	0.36
10			206	0.78	57	0.14				
11			182	0.75	44	0.10				
Total amount of dextrose eliminated, *gm.*		11.71		7.09		6.21		1.53		0.36

Body weight.

Experimental day.		Experimental day.		Experimental day.		Experimental day		Experimental day	
	kg.		*kg.*		*kg.*		*kg*		*kg*
1	2.82	1	2.36	1	2.43	1	1.84	1	2.64
6	1.92	11	1.76	11	1.68	9	1.36	9	2.03

Sugar content of blood.

	per cent		*per cent*		*per cent*		*per cent*		*per cent*
	(*a*) 0.394		(*a*) 0.295		(*a*) 0.313		(*a*) 0.260		(*a*) 0 269
1		1	(*b*) 0.308	1	(*b*) 0.408	9	(*b*) 0.357	9	(*b*) 0.357
	(*b*) 0.322		(*c*) 0.199		(*c*) 0.230		(*c*) 0.281		(*c*) 0.312
	(*a*) 0.366		(*a*) 0.328		(*a*) 0.220				
2	(*b*) 0.344	10	(*b*) 0.315	10	(*b*) 0.196				
	(*a*) 0.475		(*a*) 0.318		(*a*) 0.229				
3		11	(*b*) 0.301	11	(*b*) 0.185				
	(*b*) 0.469		(*c*) 0.160		(*c*) 0.094				
	(*a*) 0.482								
4	(*b*) 0.469								
	(*a*) 0.613								
5	(*b*) 0.499								
	(*a*) 0.449								
6	(*b*) 0.430								

TABLE II—*Concluded.*

Glycogen content of liver.				
Weight of liver, *gm*	43.6	46.2	33.7	53.0
Glycogen. *gm*	1.900	1.472	0.815	1.850
Glycogen. *per cent*	4.36	3.19	2.42	3.49
Interval between last epinephrine injection and glycogen determination, *hrs*	10.5	11.5	10	10.5
Glycogen content of muscles.				
	per cent 0.079	*per cent* 0.167	*per cent* 0.046	*per cent* 0.050

* In the last 3 days a slight albuminuria. In the other rabbits no sign of kidney injury.
(*a*), (*b*), (*c*), 2, 4, and 7 hours respectively after epinephrine injection.

amount of dextrose eliminated in 24 hours was averaged every 3 days. In cases where the number of days either in the feeding or in the fasting period could not be evenly divided by three, the last average was made from either 2 or 4 days.

TABLE III.

Glycogen Content of the Liver and Muscles of Fasted Rabbit.

Rabbit		I ♀	II ♀	III ♀	IV ♀	V ♀
Body weight.	Beginning, *kg.*	1.90	1.88	2.48	2.66	1.92
	End, *kg.*	1.46	1.30	1.83	1.84	1.56
Duration of fasting, *days*		11	10	9	8	8
Liver.	Weight, *gm.*	30.6	28.8	38.2	38.5	31.2
	Glycogen content. *gm.*	0.148	0.137	0.107	0.403	0.137
	Glycogen content. *per cent*	0.48	0.48	0.28	1.05	0.44
Glycogen content of muscles, *per cent*				0.09	0.10	0.18

DISCUSSION.

Glycosuria.—From the data in Table I and Chart I, it will be seen that there was some variation in the intensity of glycosuria after the same dose of epinephrine, both in different animals and in the same animal at different times. Repetition of epinephrine injection for a considerable length of time, however, did not materially alter the character of the curve of sugar excretion, so long as the rabbits were well nourished. These results are in harmony with those of Velich's frog experiments. When the rabbits were fasted, the intensity of glycosuria decreased markedly, although the dose of epinephrine increased in proportion relatively to the loss of body weight. This decrease of glycosuria however, never went as low as zero. It remained rather stationary at a certain stage. This does not exactly accord with Pollak's and Velich's reports. A marked decrease of glycosuria in fasting could also be seen in the rabbits which were fasted from the start of the experiments (Table II).

Hyperglycemia.—As to the epinephrine hyperglycemia, there was also some variation after the same dose of epinephrine.

In the fasting period, the intensity of hyperglycemia was sometimes a little lower than in the feeding period. In most of the cases, however, no such change could be observed. The duration of the epinephrine hyperglycemia in the fasted animals seemed also to be practically the same as in the well nourished animals.[35] These results are in harmony with those of Pollak and Bang. The question naturally arises why the intensity of glycosuria so markedly decreased in the fasting period. As no such change could be observed in a prolonged feeding period, the so called epinephrine habit, suggested by Pollak, cannot explain this. Fasting itself must be the predominant factor. Although in some cases in my experiments, the urine volume in the fasting period was markedly decreased and a slight albuminuria and cylinduria occurred (Rabbit II, Table I, Rabbit III, Table II), no severe kidney injury was ever observed; still the decreased activity of the kidneys in fasting cannot be entirely excluded. The source of sugar mobilized by epinephrine and some occasional change of hyperglycemia must also be taken into consideration. At any rate, this question still remains open.

Glycogen.—The glycogen content of the liver of the epinephrine rabbits was always greater than that of the control animals (Tables I, II, and III). This confirms Pollak's statement, but contradicts Gatin-Gruzewska's and also Agadschanianz's reports. In my epinephrine rabbits, the glycogen content of the muscles was practically the same as in the controls. This does not exactly accord with Pollak's experiments. Many authors have reported that the liver glycogen does not entirely disappear in fasting.[36] When it was desired to make an animal as glycogen-free as possible, epinephrine, phlorhizin, strychnine, cold, or forced muscle work was used in combination with fasting. In fasted hens, Nebelthau[37] observed that narcotics such as chloroform, ether, or chloral hydrate, or antipyretics can cause the storage of liver glycogen. In rabbits deprived of glycogen by fasting and strychnine, Rolly[38] observed a considerable amount of the liver

[35] Kuriyama, S., *J. Biol. Chem.*, 1917, xxix, 127.
[36] Pflüger, E., *Arch. ges. Physiol.*, 1907, cxix, 117.
[37] Nebelthau, E., *Z. Biol.*, 1891, xxviii, 138.
[38] Rolly, *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 107.

and muscle glycogen in the latter part of the fasting period, and claimed this to be the result of a new formation of glycogen from cleavage products of body protein, corresponding to the premortal increase of the nitrogen elimination. Hirsch and Rolly[39] observed a similar glycogen storage after a certain duration of infectious fever, and attributed this to a new formation of glycogen from abnormally decomposed body protein. Epstein and Baehr[40] observed that the glycogen content of the liver of fasted and phlorhizinized cats became as high as 2.18 per cent after the removal of the kidneys. The glycogen content of the muscles was similar to that of the control animals. Their explanation is that phlorhizin stimulates the liver to build up glycogen. Thus many substances which are considered to be able to mobilize the liver glycogen apparently can also cause a storage of the liver glycogen under certain experimental conditions. The glycogen formation from protein is now a generally accepted fact, and an increased protein metabolism after epinephrine injection has been reported by many investigators. In my rabbit experiments, a considerable amount of dextrose (2.9 to 17.9 gm.) was voided in the urine during the fasting period; still the glycogen content of the liver was markedly greater than that of the control animals. The glycogen content of the muscles was practically the same in both the epinephrine and control animals. It seems very possible, therefore, that unduly catabolized protein facilitated a storage of glycogen. The two rabbits which received a single epinephrine injection at the end of the fasting period (Rabbits IV and V, Table II) showed also a high glycogen content of the liver. The abnormal glycogen storage, therefore, is not due to any influence of continued epinephrine administration. In Paton's experiments an abnormal increase of protein metabolism after epinephrine injection was not large enough to account for the whole amount of sugar eliminated as originating from protein. It is, therefore, very probable that epinephrine mobilizes the previously existing carbohydrate and also induces an abnormal protein decomposition; also that the latter becomes especially apparent in fasted animals.

[39] Hirsch, C., and Rolly, *Deutsch. Arch. klin. Med.*, 1903, lxxviii, 380.
[40] Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1916, xxiv, 17.

Epinephrine, administered parenterally into the organism is believed to be destroyed very easily. When epinephrine hyperglycemia reaches its maximum or at least in the latter part of the hyperglycemia period, the epinephrine itself, which originally caused this hyperglycemia, may already be destroyed. Thus an abnormal amount of sugar circulating in the organism may be deposited as glycogen similarly to the sugar injected parenterally or taken in after digestion of carbohydrate. According to Straub[41] and Ritzmann,[42] however, glycosuria continues just as long as the continuous intravenous epinephrine injection is kept up; it disappears as soon as the injection is stopped, and further epinephrine injection makes glycosuria reappear. This accords with the phenomena found by Kretschmer[43] concerning the vasoconstrictive effect of epinephrine. The only difference is that the glycosuric effect of epinephrine needs a much longer latent period than its vasoconstrictive effect. The course of the hyperglycemia was not studied here. If the epinephrine hyperglycemia really continues eactly as long as an excess of epinephrine exists in the circulation, the sugar mobilized by epinephrine may have no chance to be deposited again. Notwithstanding Straub and Ritzmann's statements, this possibility for a new formation of glycogen cannot be entirely excluded.

In explaining an abnormal glycogen formation after epinephrine injection, it may be said that epinephrine exerts a specific effect upon the liver, making this organ build up glycogen. It may act directly upon the liver or indirectly through some other endocrine organs. Mackenzie[44] is inclined to believe such a specific effect. As discussed above, however, this does not seem to be a necessary assumption. Many other toxic substances can behave in a somewhat comparable manner. The reason why the glycogen content of the muscles did not increase like that of the liver may be well explained by the fact that the liver can store glycogen much easier than the muscles.

[41] Straub, W., *Münch. med. Woch.*, 1909, lvi, 493.
[42] Ritzmann, H., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 231.
[43] Kretschmer, W., *Arch. exp. Path. u. Pharmakol.*, 1907, lvii, 423.
[44] Mackenzie, G. M., *Arch. Int. Med.*, 1917, xix, 593.

SUMMARY.

Daily repetition of subcutaneous injection of epinephrine does not materially alter the character of the curve of sugar excretion, so long as the animals are kept in a good nutritional condition. When the animals are submitted to fasting, the intensity of the epinephrine glycosuria decreases markedly, and remains rather stationary at a certain level.

Epinephrine hyperglycemia is not markedly modified by daily repetition of epinephrine injection in well nourished animals. In the fasting period, the intensity of hyperglycemia sometimes becomes a little lower; in most of the cases, however, it is as high as in the feeding period.

The marked diminution of epinephrine glycosuria in fasting therefore, cannot be explained by insufficient hyperglycemia or by the so called epinephrine habit. Fasting must be the predominant factor. An adequate explanation is still lacking. The mechanism of hyperglycemia in fasted animals, the decreased activity of the kidneys in fasting, and some occasional change of hyperglycemia must be taken into consideration.

The livers of the rabbits which are fasted and submitted to daily epinephrine injection, contain a much larger amount of glycogen than those of merely fasted animals. The glycogen content of the skeletal muscles shows no notable difference in either case. A single injection of epinephrine into fasted rabbits can also cause a similar increase of the glycogen content of the liver. The abnormal glycogen storage in the liver, therefore, is not due to any influence of the continued epinephrine administration. Though some investigators suggest that epinephrine exerts a specific effect upon the liver, making this organ build up glycogen, acting directly upon the liver or indirectly through some other endocrine organs, this assumption does not seem to be absolutely necessary. Many other toxic substances can behave in a somewhat comparable manner. A new formation of glycogen from the unduly catabolized protein must be taken into consideration. Furthermore, a possibility that the sugar first mobilized by epinephrine is deposited again after the epinephrine is destroyed in the organism, cannot be entirely excluded.

Though some investigators use a combination of fasting and epinephrine injection in order to deprive the organism of glycogen, such a procedure fails to accomplish the desired end.

THE ADRENALS IN RELATION TO CARBOHYDRATE METABOLISM. II.

THE INFLUENCE OF ADRENALECTOMY UPON THE GLYCOGENETIC POWER OF THE LIVER.

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Porges[1] and Bernstein[2] reported a lowering of the blood sugar in Addison's disease. Porges showed also that the blood sugar in adrenalectomized dogs falls regularly during the time that the animals survive. The experiments of Bierry and Malloizel[3] accord with these observations. They found that the concentration of sugar in the blood, after extirpation of the adrenals of dogs, fell to one-half or four-fifths of the original amount. Schirokauer[4] reported that in Addison's disease hypoglycemia is not always observed, and is not an important symptom. In Frank and Isaac's rabbit experiments,[5] the blood sugar content after adrenalectomy was normal in five cases, decreased in two cases. They concluded that adrenalectomy does not necessarily bring about hypoglycemia. They argued that hypoglycemia found in adrenalectomized dogs, which were already in dying condition, could not be accepted in drawing conclusions. In Nishi's rabbit experiment,[6] the blood sugar content after adrenalectomy remained within the physiological variation. Frouin[7] performed pancreatectomy in dogs, from which one adrenal and two-thirds of the other had previously been removed, and found that the intensity of glycosuria was less than in those from which the pancreas alone had been removed. Though Mayer[8] found that hyperglycemia or glycosuria of depancreatized animals was sometimes decreased by removal of the adrenals, the rapid death of the animals after the removal of the adrenals did not allow him to draw any

[1] Porges, O., *Z. klin. Med.*, 1909, lxix, 341.

[2] Bernstein, S., *Berl. klin. Woch.*, 1911, xlviii, 1794.

[3] Bierry, H., and Malloizel, L., *Compt. rend. Soc. biol.*, 1908, lxv, 232.

[4] Schirokauer, H., *Berl. klin. Woch.*, 1911, xlviii, 1505.

[5] Frank, E., and Isaac, S., *Z. exp. Path. u. Therap.*, 1910, vii, 326.

[6] Nishi, M., *Arch. exp. Path. u. Pharm.*, 1909, lxi, 186.

[7] Frouin, A., *Compt. rend. Soc. biol.*, 1908, lxiv, 216.

[8] Mayer, A., *Compt. rend. Soc. biol.*, 1908, lxiv, 219.

positive conclusion. Mackenzie[9] also reported that the blood sugar content of depancreatized animals can be decreased by the removal of the adrenals.

Schwarz,[10] using rats, determined the glycogen content of the liver after both adrenals had been extirpated and found that after bread feeding the liver of these animals contained very little or no glycogen. An addition of levulose, starch, or animo-acids to the bread diet failed to induce a glycogen storage in the liver. Only when glucose or sucrose was added was a considerable amount of liver glycogen found. He concluded that the disappearance of the liver glycogen in his rats was not due to the marasmus, but was a specific deficiency phenomenon after the removal of the adrenals. The decrease of the liver glycogen after removal of both adrenals of dogs was also reported by Porges.[11] His animals were killed from 4 to 9 hours after the adrenalectomy. Kahn and Starkenstein[12] reported that they confirmed Schwarz's statement. In their two experiments, one adrenalectomized albino rat contained no liver glycogen, but the glycogen content of the liver of the other operated rat was as high as 2.38 per cent. Though in his control rat the glycogen content of the liver was 17.7 per cent, this percentage must be considered as unusually high in comparison to Schwarz's control animals. The glycogen content of Schwarz's normal rats was from 2.44 to 5.07 per cent. Kahn and Starkenstein found also that in rabbits the glycogen storage of the liver remains normal after removal of both adrenals. Confirming Porges' statement,[11] they observed further that in a dog the liver glycogen disappeared after adrenalectomy; they attributed this, however, to the serious operation. After administration of large quantities of glucose intravenously or by the alimentary tract to adrenalectomized dogs, Mackenzie[9] found that little or no glycogen was demonstrable in the liver cells when stained by Best's method. He considers that epinephrine performs a double function, as far as glycogen metabolism is concerned; namely, it is necessary for glycogenesis, and when present in excess, it stimulates glycogenolysis. As to the fact that epinephrine injection into fasted animals causes a glycogen storage in the liver, a discussion will be found in the preceding paper.[13]

In regard to the question whether the adrenal or epinephrine really has a specific effect upon the glycogenesis of the liver, I have repeated Schwarz's experiments.

[9] Mackenzie, G. M., *Arch. Int. Med.*, 1917, xix, 593.

[10] Schwarz, O., *Arch. ges. Physiol.*, 1910, cxxxiv, 259.

[11] Porges, *Z. klin. Med.*, 1910, lxx, 243.

[12] Kahn, R. H., and Starkenstein, E., *Arch. ges. Physiol.*, 1911, cxxxix, 181.

[13] Kuriyama, S., *J. Biol. Chem.*, 1918, xxxiv, 269.

Methods.

Medium sized albino rats, both male and female, were used. Adrenalectomy was performed at two different times, the interval between the two operations varying from about 2 to 7 weeks. Owing to the anatomical position, adrenalectomy is very easy in rats. Ether was used as an anesthetic. Through a small cut in the side below the costal arch, the adrenal was removed transperitoneally. The glands were always extirpated, together with some surrounding tissues, without disintegrating them. The animals recovered from the operation very quickly. After the second adrenalectomy, the animals gradually became weak, ate less, lost in body weight, and, if not used for further experiments, usually died in from 7 to 15 days, autopsy showing no sign of infection. For studying the glycogenetic function of the liver, it was very desirable to deprive the animal as far as possible of the previously existing glycogen. The weakness of the animals, however, prevented me from carrying through this procedure too strenuously. In some experiments a few days of fasting were inserted before feeding them on the desired food. As food after the second adrenalectomy, various substances were used, alone or in different combinations. The food was usually not made in the form of paste. 10 gm. of the desired food were put in the cage every day. When a small amount of it had been scattered by the animals, as much of the residue as possible was collected in estimating the amount of food eaten. On the last experimental day, the food was given so that it would be eaten within 12 hours before glycogen determination. The food, designated as dog biscuit-lard-diet, consists of 30 parts of lard and 70 parts of dog biscuit. At the end of the feeding period, the animals were killed by decapitation and the liver glycogen was determined by Pflüger's method. 10 cc. of 60 per cent potassium hydroxide solution were first kept in a steam bath. The liver, cut in pieces, was thrown into the hot alkali solution together with a certain volume of water, which corresponded to the difference of the liver weight and 10 gm. The glycogen finally obtained was hydrolyzed with hydrochloric acid and the sugar thus produced was first determined by Allihn's gravimetric method, but later the Pavy-Kumagawa-Suto method was employed. As controls the liver glycogen of the rats from

TABLE I.

Influence of Bilateral Adrenalectomy upon the Glycogenetic Power of the Liver.

Group A. Food: sucrose, alone or combined with other food substances.

Rat		I ♀	II ♀	III ♀	IV ♂	V ♂
First adrenalectomy	Body weight, *gm.*	139.8	89.6	126.7	274.1	203.2
	Side	Left.	Right.	Left.	Left.	Left.
Interval between 1st and 2nd adrenalectomy, *days*		36	34	36	29	30
Second adrenalectomy	Body weight, *gm.*	156.1	113.8	152.8	262.3	181.9
Interval between 2nd adrenalectomy and glycogen determination, *days*		3	3	3	5	4
Amount of food in the last few days of experiment, *gm.*	1st day	Fasted.	Fasted.	Fasted.	Dog biscuit and oats freely.	Dog biscuit and oats freely.
	2nd "	"	"	"	" " "	Fasted.
	3rd "	Sucrose, 2.4	Sucrose, 1.7	Sucrose, 2.2	Fasted.	"
	4th "				"	⅔ sucrose ⅓ dog biscuit } 7.8
	5th "				⅔ sucrose ⅓ dog biscuit } 3.7	
Body weight at end of experiment, *gm.*		142.0	88.0	128.6	240.8	170.2
Liver.	Weight, *gm.*	5.8	4.1	4.3	7.0	6.9
	Glycogen content { *mg.*	158.0	128.0	63.0	325.0	120.0
	{ *per cent.*	2.72	3.12	1.47	4.64	1.74

Group B. Food: levulose or lactose, alone or combined with other food substances.

Rat		I ♂	II ♂	III ♂	IV ♂	V ♂	VI ♀	VII ♂
First adrenalectomy.	Body weight, *gm.*	290.6	377.0	175.8	174.6	110.7	99.8	268.5
	Side	Right.	Left.	Left.	Left.	Right.	Right.	Right.
Interval between 1st and 2nd adrenalectomy, *days*		24	40	24	30	50	50	29
Second adrenalectomy.	Body weight, *gm.*	295.2	345.6	167.9	184.5	152.9	129.0	309.2
Interval between 2nd adrenalectomy and glycogen determination, *days*		4	2	3	4	4	4	4
Amount of food in the last few days of experiment, *gm.*	1st day	Fasted.	Fasted.	Fasted.	Dog biscuit and oats freely.	Dog biscuit and oats freely.	Dog biscuit and oats freely.	Dog biscuit and oats freely.
	2nd "	Levulose, 2.0	½ levulose, ½ casein } 3.7	½ levulose, ½ casein } 1.2	Fasted.	" "	" "	" "
	3rd "	" 1.5		½ levulose, ½ casein } 2.8	"	" "	" "	" "
	4th "	" 1.1			⅔ levulose, ⅓ dog biscuit } 8.7	¼ lactose, ¾ dog biscuit } 4.2	¼ lactose, ¾ dog biscuit } 4.5	¼ lactose, ¾ dog biscuit } 4.5
Body weight at end of experiment, *gm.*		254.6	314.8	147.7	178.0	136.0	118.6	273.4
Liver.	Weight, *gm.*	6.6	9.1	5.7	6.6	4.9	4.6	7.3
	Glycogen content. *mg*	11.0	34.0	143.5	215.5	40.0	76.0	61.0
	Glycogen content. *per cent*	0.17	0.37	2.52	3.27	0.82	1.65	0.84

TABLE I.—*Concluded.*

Group C. Food: starch or its cleavage products, alone or combined with other food substances.

Rat		I ♂	II ♂	III ♂	IV ♂	V ♀	VI ♀	VII ♂
First adrenalectomy.	Body weight, *gm.*	250.2	208.6	292.2	183.4	101.1	100.0	92.5
	Side	Right.	Left.	Right.	Right.	Right.	Right.	Right.
Interval between 1st and 2nd adrenalectomy, *days*		40	24	24	29	50	49	50
Second adrenalectomy.	Body weight, *gm.*	268.0	232.5	303.1	228.2	129.0	134.1	139.5
Interval between 2nd adrenalectomy and glycogen determination, *days*		3	3	3	4	3	3	3
Amount of food in the last few days of experiment, *gm.*	1st day	Fasted.	Fasted.	Fasted.	Dog biscuit and oats freely.	Dog biscuit and oats freely.	Fasted.	Dog biscuit and oats freely.
	2nd "	½ starch, ½ levulose } 9.3	½ starch, ½ casein } 5.4	½ starch, ½ casein } 13.1	" " "	" " "	⅔ dextrin, ⅓ maltose } 4.2	⅜ starch, ⅖ dextrin, ⅙ maltose } 6.6
	3rd "	½ starch, ½ levulose } 9.3	½ starch, ½ casein } 5.1	½ starch, ½ casein } 7.2	" " "	⅔ starch, ⅓ dextrin, ⅙ maltose } 5.5	⅔ dextrin, ⅓ maltose } 4.5	
	4th "				⅜ starch, ⅖ dextrin, ⅙ maltose } 6.2			
Body weight at end of experiment, *gm.*		246.1	209.0	280.9	203.7	124.7	125.7	130.6
Liver.	Weight, *gm.*	7.0	7.8	8.4	7.3	4.0	4.2	4.8
	Glycogen content, *mg.*	220.7	58.6	91.0	6.1	7.8	69.5	105.0
	Glycogen content, *per cent*	3.15	0.75	1.08	0.84	1.95	1.65	2.19

Group D. Food: dog biscuit-lard-diet.

Rat		I ♀	II ♀	III ♀	IV ♀	V ♀	VI ♂	VII ♂	VIII ♂	IX ♂
First adrenalectomy.	Body weight, *gm*	296.1	212.5	268.4	248.6	203.0	309.0	278.9	237.6	327.7
	Side	Right.	Right.	Left.	Left.	Left.	Right.	Right.	Right.	Right.
Interval between 1st and 2nd adrenalectomy, *days*		16	16	11	13	13	14	14	14	13
Second adrenalectomy.	Body weight, *gm*	289.8	214.3	259.9	254.2	227.9	328.2	318.7	272.2	311.9
Interval between 2nd adrenalectomy and glycogen determination, *days*		5	5	7	4	4	6	6	6	6
Amount of food in the last few days of experiment. Dog biscuit-lard-diet, *gm.*		Freely for 2 days.	Freely for 2 days.	Freely for 4 days.	Freely for 2 days.	Freely for 2 days.	Freely for 2 days.	Freely for 2 days.	Freely for 2 days.	Freely for 2 days.
	1st day	3.1	1.7	0.5	10.0	0.9	10.0	10.0	2.8	3.1
	2nd "	3.9	2.5	2.1	9.3	1.9	10.0	8.7	0.1	2.9
	3rd "	2.0	1.2	0.8			6.9	7.1	0	3.1
	4th "						0.5	3.8	0	0
Body weight at end of experiment, *gm*		260.6	186.8	232.8	245.9	209.0	297.7	287.2	228.9	260.5
Liver.	Weight, *gm*	10.1	6.0	5.0	8.3	5.4	8.4	10.0	6.5	9.3
	Glycogen content, *mg*	30.2	Trace.	Trace.	295.0	Trace.	Trace.	285.2	0	Trace
	Glycogen content, *per cent*	0.30			3.55			2.85		
Blood sugar content, *per cent*		0.112	0.120	0.083	0.089	0.099	0.080	0.103	0.080	0.112

TABLE II.

Glycogen Content of the Liver of Rats, from Which One Adrenal Only Was Removed. Control Experiments.

Rat		I ♀	II ♀	III ♀	IV ♂	V ♂	VI ♀
Adrenalectomy.	Body weight, *gm.*	212.8	229.0	238.3	283.2	236.6	109.9
	Side	Right.	Right.	Left.	Right.	Right.	Right.
Interval between operation and glycogen determination, *days*		13	12	9	44	44	89
Food after adrenalectomy. Dog biscuit-lard-diet		Freely for 10 days.	Freely for 9 days.	Freely for 6 days.	Freely for 42 days.	Freely for 42 days.	Freely for 87 days.
Amount of food in the last few days of experiment. Dog biscuit-lard-diet, *gm.*	1st day	8.0	7.9	5.3	10.0	10.0	10.0
	2nd "	6.3	10.1	6.4	10.0	10.0	10.0
	3rd "	6.0	6.0	8.4			
Body weight at end of experiment, *gm.*		196.8	209.7	222.9	279.4	265.8	150.3
Liver.	Weight, *gm.*	6.7	8.4	8.2	10.6	9.3	6.0
	Glycogen content. *mg.*	227.4	554.0	421.0	875.0	855.1	268.9
	Glycogen content. *per cent.*	3.39	6.60	5.13	8.25	9.20	4.48
Blood sugar content, *per cent*		0.100	0.121	0.111	0.130	0.120	0.112

which only one adrenal had been removed was estimated. Desiring to find out whether the often reported hypoglycemia in adrenalectomized animals can be observed in rats, the blood in some cases was collected at the time of bleeding, and analyzed for the sugar content. The Lewis-Benedict method was used. The results are detailed in Tables I and II.

From these tables, it will be seen that the liver of the rats, from which both adrenals had been removed, did not always show a marked decrease of the liver glycogen. Most of the animals which showed the complete loss or a marked decrease of the liver glycogen were those which had taken no food or a relatively small amount. If these results are compared with the data concerning the glycogenetic power of fasted rats[14] or those from which one adrenal only had been removed, many of the rats without both adrenals show some difficulty in storing glycogen after taking a certain amount of food which would otherwise be enough to induce a marked glycogen storage. Among the food substances which were given after the second adrenalectomy, sucrose was the most effective in storing the liver glycogen. Many other food substances, including mono-, di-, and polysaccharides, and protein, used alone or in various combinations, however, could also induce a storage of a considerable amount of the liver glycogen. Schwarz[10] argues that glucose only can be used in forming glycogen. This does not accord with my results. Schwarz reported that in fifteen rats which were fed on *Semmel* after the second adrenalectomy and killed in from 3 to 36 days, only one bore traces of the liver glycogen and all the others showed none. The amount of the food taken is not stated. Some other adrenalectomized rats, however, which were not used for glycogen determination, were able to gain in body weight. The amount of levulose, starch, and amino-acids, used by him, seems to be too small for such investigations. He stated that his animals were not as lively as before the operation. A probable decrease of the activity of the alimentary tract due to the general weakness must be taken into consideration. As his adrenalectomized rats fed on *Semmel* diet could live for a long time, the amount of food was probably not very small. According to him, in sucrose feeding the glucose component only was used for

[14] Kuriyama, *J. Biol. Chem.*, 1918, xxxiii, 197.

glycogen formation; on the other hand, in *Semmel* diet, the chief constituent, starch, which can produce glucose after digestion, could not be used for glycogen formation. Though the composition of the *Semmel* used by him is not stated, the bread usually contains a small amount of sugar. According to König,[15] various kinds of bread, manufactured in different places, contained sugar in proportion of 2, 3, 4, or much more per cent. In examining the extracts of a few samples of table bread and rolls, I also found a strong reducing power. Contrary to Schwarz's statement, in Mackenzie's experiment[9] even glucose failed to form glycogen in the liver of an adrenalectomized dog. The details of his experiments, including the general conditions of the animals, however, are not stated in his preliminary report.

At any rate, in my experiments, no evidence which positively indicates any specific influence of adrenalectomy upon the glycogenetic power of the liver could be obtained. The emaciated condition of the adrenalectomized rats seems to be sufficient in explaining some difficulty in storing the liver glycogen.

The blood sugar content of the adrenalectomized rats varied between 0.12 to 0.08 per cent (Table I, Group D). On an average, it was a little lower than that of the control animals (Table II), still remaining within the physiological variation, however. These results are in harmony with those of Frank and Isaac and Nishi.

SUMMARY.

The adrenals of medium sized albino rats were removed at two different times and after the second adrenalectomy, the animals were fed on various kinds of food. The liver of these animals contained a considerable amount of glycogen if the amount of food taken was of a sufficient quantity. In comparing with the data concerning the glycogenetic power of the fasted rats, or those from which one adrenal only had been removed, many of the rats without both adrenals showed some difficulty in storing glycogen after taking a certain amount of food which would otherwise be enough to induce a marked glycogen storage. The emaciated condition

[15] König, J., Chem. menschl. Nahrungs- u. Genussmittel, Berlin, 4th edition, 1903, i, 671: 1904, ii, 878.

of the adrenalectomized rats seems to be a sufficient reason for explaining this difficulty. Among the food substances which were given after the second adrenalectomy, sucrose was the most effective in storing liver glycogen. Many other food substances, including mono-, di-, and polysaccharides and protein, used alone or in various combinations, however, could also induce a storage of a considerable amount of the liver glycogen. In my experiments, therefore, no evidence which positively indicates any specific influence of adrenalectomy upon the glycogenetic power of the liver could be obtained. As far as concerns the glycogen storage of the liver, the rat acts about the same as the rabbit, after adrenalectomy.

The blood sugar content of the adrenalectomized rats was, on the average, a little lower than that of the control animals, still remaining within the physiological variation. This is in harmony with the rabbit experiments by Frank and Isaac and Nishi.

THE ADRENALS IN RELATION TO CARBOHYDRATE METABOLISM. III.

THE EPINEPHRINE CONTENT OF THE ADRENALS IN VARIOUS EXPERIMENTAL CONDITIONS.

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Since the discovery that epinephrine injection induces a marked hyperglycemia and glycosuria, many forms of hyperglycemia and glycosuria, produced in certain diseases or experimental conditions, have been reported to be due to an excess of epinephrine in the organism.[1] For example, hyperglycemia and glycosuria after *piqûre*, in caffeine, morphine, strychnine, chloroform, urethane, or carbon monoxide intoxication, as a result of asphyxia, after injection of saline solution, after irritation of sensory nerves, the so called emotional glycosuria, and that invoked by cold, at times have all been explained in this manner. In some of these examples, however, many investigators dispute the alleged relationship of epinephrine.

Changes in the medulla of the adrenals have been reported in various experimental conditions. Thus Kahn[2] found a decrease of the epinephrine content of the adrenals, estimated both by the chromaffin reaction and the vasoconstrictive effect, after *piqûre*. This was confirmed by Quinquaud.[3] Jarisch,[4] however, claims that *piqûre* does not cause any demonstrable histological changes in the medulla of the adrenals, and that a decrease of the chromaffin substance of one adrenal, found after the removal of the companion gland and *piqûre* is due only to the serious operation. Starkenstein[5] reported that in CO poisoning or asphyxia, the epinephrine content of the adrenals is reduced, this change, however, being prevented by section of the splanchnic nerve. His findings are not in harmony with Stewart

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[1] References cited by Bang, I., Der Blutzucker, Wiesbaden, 1913, 84. Bayer, G., *Ergebn. allg. Path.*, 1911, xiv, pt. 2, 1

[2] Kahn, R. H., *Arch. ges. Physiol.*, 1911, cxl, 209.

[3] Quinquaud, A., Collège de France, Paris, 1913-15, iii.

[4] Jarisch, A., *Z. exp. Path. u. Therap.*, 1913, xiii, 520.

[5] Starkenstein, E., *Z. exp. Path. u. Therap.*, 1911, x, 78.

and Rogoff's experiments.[6] Schur and Wiesel[7] and Ingier and Schmorl[8] observed that narcosis causes the adrenals to lose their epinephrine store, either partly or entirely. Shiota,[9] Kahn,[10] and Borberg[11] failed to confirm this. Biedl[12] reported that the amounts of chromaffin and vasoconstrictive substance in the adrenals do not always run parallel. For example, the adrenals which showed a marked decrease of the chromaffin substance, still had an almost unchanged vasoconstrictive effect. A similar divergence between the chromaffin reaction and Comessatti's reaction was reported by Ingier and Schmorl.[8] Battelli and Boatta[13] observed a marked decrease of the epinephrine content of the adrenals after strenuous muscle work. This was confirmed by Schur and Wiesel,[14] but not by Kahn.[10] In Schur and Wiesel's experiments, notwithstanding a decrease of the epinephrine content of the adrenals, no glycosuria was observed. They attributed this to a deficient glycogen storage. Hyperemia and degenerative changes of the adrenals in various infections or intoxications with bacterial toxins are well known. Luksch[15,16] reported that the adrenals lost the vasoconstrictive substance, either partly or entirely, in diphtheria intoxication or by infection with tubercle or staphylococcus bacilli. In diphtheria intoxication the vasoconstrictive effect of the adrenal extract was lost, notwithstanding the presence or absence of visible changes. His experiments show, further, that the adrenals are affected similarly in uremia, or phosphorus poisoning; but such is not the case in fasting or in fever after heat puncture. Luksch's findings in diphtheria intoxication were confirmed by Ritchie and Bruce[17] and Elliott.[18] Estimating the epinephrine content of the serum of the blood, taken from the adrenal vein, by the frog eye method, Erhmann[19] reported that notwithstanding marked anatomical changes of the adrenals in diphtheria intoxication, there was no diminution in the amount of epinephrine, but rather an increase. In morphine intoxication Elliott[18] reported that when the splanchnic nerve innervating one adrenal had previously been cut, the epinephrine content of this adrenal was not decreased as much as that of the

[6] Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1917, x, 49.

[7] Schur, H., and Wiesel, J., *Wien. klin. Woch.*, 1908, xxi, 247.

[8] Ingier, A., and Schmorl, G., *Deutsch. Arch. klin. Med.*, 1911, civ, 125.

[9] Shiota, H., *Arch. ges. Physiol.*, 1909, cxxviii, 431.

[10] Kahn, *Arch. ges. Physiol.*, 1909, cxxviii, 519.

[11] Borberg, N. C., *Skand. Arch. Physiol.*, 1913, xxviii, 91.

[12] Biedl, A., Innere Sekretion, Berlin and Wien, 1910, 230.

[13] Battelli, F., and Boatta, G. B., *Compt. rend. Soc. biol.*, 1902, liv, 1203.

[14] Schur and Wiesel, *Wien. klin. Woch.*, 1907, xx, 1202.

[15] Luksch, F., *Wien. klin. Woch.*, 1905, xviii, 345.

[16] Luksch, *Berl. klin. Woch.*, 1909, xlvi, 1979.

[17] Ritchie, J., and Bruce, A. N., *Quart. J. Exp. Physiol.*, 1911, iv, 127.

[18] Elliott, T. R., *J. Physiol.*, 1912, xliv, 374.

[19] Ehrmann, R., *Arch. exp. Path. u. Pharm.*, 1906, lv, 39.

companion adrenal. Similar results were obtained also in diphtheria intoxication. On the other hand, Stewart and Rogoff[20] observed that animals which had had one adrenal removed and the splanchnic nerve innervating the companion gland cut, could still react with hyperglycemia to asphyxia, or ether, without losing the epinephrine storage of the remaining adrenal.

Hyperglycemia and glycosuria which are reported to be due to an excess of epinephrine, sometimes correspond well to the findings of the adrenals; for example, in *piqûre*, asphyxia, poisoning with carbon monoxide, morphine, or narcotics. Many contradictory reports,[4-6,20,21] however, make one hesitate to accept a necessary relation between hyperglycemia and increased secretion of epinephrine, in all these conditions.

In connection with the studies of the adrenals in relation to carbohydrate metabolism, I have made some investigations concerning the epinephrine content of the adrenals in various experimental conditions.

Method of Estimating the Epinephrine Content of the Adrenals.

Rabbits and albino rats were used. Epinephrine content of the adrenals was determined by the Folin-Cannon-Denis method.[22] In rat experiments, the two adrenals were extracted in a 10 cc. volumetric flask,[23] while in rabbit experiments each gland was separately examined and a 25 cc. volumetric flask was used for extraction. There seems to be no clear statement whether the mode of killing experimental animals has any bearing upon the epinephrine content of the adrenals or not. In my experiments, the rabbits were made unconscious by a blow on the neck and then bled. The rats were killed by bleeding.

[20] Stewart and Rogoff, *Am. J. Physiol.*, 1917, xliv, 543.

[21] Trendelenburg, P., and Fleischhauer, K., *Z. ges. exp. Med.*, 1913, i, 369, abstr. in *Jahrsbr. Tierchem.*, 1914, xliv, 339. Freund, H., and Marchand, F., *Arch. exp. Path. u. Pharm.*, 1914, lxxvi, 324. Wertheimer, E., and Battez, G., *Compt. rend. Soc. biol.*, 1914, lxxvi, 617.

[22] Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 477.

[23] Kuriyama, S., *J. Biol. Chem.*, 1918, xxxiii, 207.

Postmortem Change of the Epinephrine Content of the Adrenals.

In relation to the fate of epinephrine injected into the organism, a quick oxidative destruction of epinephrine in the tissue, especially in the liver,[24] an important rôle of alkali in destroying epinephrine in the blood,[25] a prolongation of the vasoconstrictive effect of epinephrine by a simultaneous injection of acid,[26] and other factors have been reported. As to the postmortem change of the epinephrine content of the adrenals, Kohn and Wiesel[27] reported that the chromaffin substance of the adrenals had already disappeared from 12 to 30 hours after death. According to Comessatti,[28] the epinephrine content of the adrenals decreases in correspondence to the postmortem softening of the medulla. Lukseh[15] and Ingier and Schmorl,[8] however, could not confirm a rapid disappearance of the chromaffin substance of the adrenals after death. The latter investigators observed a strong chromaffin reaction from 48 to 72 hours after death; in the adrenals of animals in a slaughter house even 5 days after death, and also in the adrenals whose cortex had already been putrefying. They obtained similar results in human adrenals, extirpated and conserved at 8°C. Dividing the ground adrenal into two portions and estimating their epinephrine content at different times, they found that epinephrine is destroyed somewhat on standing, especially if the material is in contact with air. As preliminary experiments, I have carried through some investigations concerning this problem.

Method.—The fairly similar content of epinephrine in both adrenals of dogs, rabbits, and cats was reported by Battelli and Ornstein,[29] Elliott,[18] Stewart and Rogoff.[30] In my own experiments this has been confirmed as will be seen later. If the epinephrine content of one adrenal is determined immediately and that of the companion gland after a certain length of time and there exists a marked difference between these data, this difference can be attributed to postmortem changes. In my experiments, both adrenals of a normal rabbit, killed by a blow on the neck and bleeding, were extirpated aseptically. Special care was taken not to

[24] Langlois, P., *Compt. rend. Soc. biol.*, 1897, xlix, 524, 571. Athanasiu and Langlois, *ibid.*, 575.

[25] Embden, G., and von Fürth, O., *Beitr. chem. Physiol. u. Path.*, 1903, iv, 421.

[26] Kretschmer, W., *Arch. exp. Path. u. Pharmakol.*, 1907, lvii, 438.

[27] Kohn and Wiesel, cited by Biedl, A., Innere Sekretion, Berlin and Wien, 1910, 230.

[28] Comessatti, G., *Arch. exp. Path. u. Pharmakol.*, 1910, lxii, 190.

[29] Battelli, F., and Ornstein, S., *Compt. rend. Soc. biol.*, 1906, lxi, 677.

[30] Stewart and Rogoff, *J. Exp. Med.*, 1916, xxiv, 709.

damage the shape of the gland. After weighing, one was analyzed immediately for its epinephrine content; the other being kept for 24 to 68 hours at room temperature. When it was desired to keep the latter in the air, the gland was placed in a sterilized weighing bottle (capacity 8 cc.). When an oxygen-free medium was desired, it was kept in a specially devised chamber, from which oxygen was absorbed by pyrogallic acid and a 10 per cent sodium hydroxide solution. The outer surface of the alkaline solution was covered with a thick layer of mineral oil. This chamber consisted of one small cylinder (8 cc.) and two beakers (60 and 30 cc.). The cylinder, with some glass wool, was placed in the larger beaker and covered with the smaller one. The adrenal was put on the glass wool in the cylinder. Before determining the epinephrine content of this gland at the end of a desired length of time, the sterility was tested with agar-agar culture media. Folin and his coworkers[22] stated that substances, which are usually contained in the animal tissues and react to the phosphotungstic acid solution with the same color as epinephrine, are negligible in the determination of epinephrine in the adrenals, their chemical method giving exactly the same value as tested by the physiological method. Lewis[31] reported that though Folin's method is serviceable for the determination of epinephrine in the adrenals, it cannot be employed for the adrenals of the fetus, on account of the high content of uric acid in the latter. If the adrenal is kept for some time, uric acid or some other substances which disturb the epinephrine determination may be increased. When the salivary gland or the spleen of normal rabbits was manipulated the same as for the epinephrine determination of the adrenals by Folin's method, the blue color produced by the phosphotungstic acid solution was as strong as if the material contained epinephrine in proportion of 0.1 to 0.15 mg. per gm. of the tissue. The intensity of the blue color produced by the phosphotungstic acid solution, however, was not materially increased by storing the testing materials under experimental conditions employed. The results are shown in Table I.

From this table, it will be seen that the epinephrine content of the adrenals extirpated without damaging their form and

[31] Lewis, J. H., *J. Biol. Chem.*, 1916, xxiv, 249.

TABLE I.

Postmortem Change of Epinephrine Content of Adrenals, Extirpated without Damage to Their Form and Kept Aseptically.

Rabbit			I ♀	II ♀	III ♀	IV ♀	V ♀	VI ♂
Body weight, *kg*			1.46	1.94	1.90	2.00	2.40	1.90
Left adrenal.	Weight, *mg*		149.6	230.9	243.0	198.5	259.9	157.5
	Mode of keeping		In oxygen-free medium for 50 hrs.	Examined immediately.	In air for 67 hrs.	Examined immediately.	In oxygen-free medium for 68 hrs.	In air for 65 hrs.
	Epinephrine content.	*mg*	0.271	0.379	0.104	0.276	0.374	0.084
		Per gm. of gland, *mg*	1.81	1.64	0.43	1.39	1.44	0.53
Right adrenal.	Weight, *mg*		138.5	200.1	202.0	153.3	210.4	138.1
	Mode of keeping		Examined immediately.	In oxygen-free medium for 67 hrs.	Examined immediately.	In air for 48 hrs.	In air for 68 hrs.	In oxygen-free medium for 65 hrs.
	Epinephrine content.	*mg*	0.230	0.337	0.204	0.152	0.110	0.235
		Per gm. of gland, *mg*	1.66	1.68	1.01	0.99	0.52	1.70

kept in air at room temperature, is markedly decreased in the course of a few days. When the gland was kept in the oxygen-free medium, no such decrease was observed. The diversity of the results of the previous investigators, therefore, is probably due to the difference in the methods of keeping the glands. In the following experiments, the epinephrine content of the adrenals was determined as quickly as possible, with a minimum exposure to the air.

Epinephrine Content of the Adrenals after Subcutaneous Injection of Epinephrine.

Caussade[32] observed the hypertrophy of the adrenals after injecting adrenal extract repeatedly for 1 to 4 months. Vincent[33] could not confirm this. Elliott[18,34] noticed that epinephrine injection does not change the epinephrine content of the adrenals. The decreased content in his experiments was considered to be due only to anesthesia. Injecting epinephrine intravenously or intraperitoneally, Borberg[11] found that the chromaffin substance of the adrenals was sometimes decreased, sometimes of normal content, and argued that the chromaffin substance of the adrenals is not directly affected by the intravenous injection of epinephrine.

The epinephrine secretion of the adrenals is controlled by the sympathetic nerve, and epinephrine stimulates the sympathetic nerve system. In order to obtain more data concerning the influence of epinephrine upon the adrenals, determination has been made of the epinephrine content of the adrenals of rabbits after daily epinephrine injection for a considerable length of time and also after an acute intoxication with epinephrine. The results are detailed in Tables II and III. Rabbits I to VII in Table II are the same animals used in Paper I (Rabbits I to V in Table I and Rabbits II and III in Table II[35]). All the animals which were submitted to daily repetition of epinephrine injection (Table II) were fasted for 8 to 14 days before they were killed for determination of the epinephrine content of the adrenals. As to the influence of fasting upon the epinephrine content of the adrenals, Luksch[15] reported that fasting did not affect

[32] Caussade, G., *Compt. rend. Soc. biol.*, 1896, xlviii, 67.
[33] Vincent, S., *J. Physiol.*, 1897–98, xxii, 111.
[34] Elliott, *J. Physiol.*, 1904–05, xxxii, 401.
[35] Kuriyama, *J. Biol. Chem.*, 1918, xxxiv, 269.

TABLE II.

Influence of Repetition of Epinephrine Injection upon the Epinephrine Content of the Adrenals.

Rabbit		I ♀	II ♀	III ♀	IV ♀	V ♀	VI ♀	VII ♀
Body weight.	Beginning, *kg*.	2.12	1.88	2.28	2.11	2.38	2.36	2.43
	End, *kg*.	1.36	1.55	1.74	1.40	1.30	1.76	1.68
Amount of epinephrine, injected daily, per kilo of initial body weight, *mg*		0.75	0.5	1.0	1.0	1.0	1.0	0.75
Duration of experiment, *days*.		23	29	52	41	25	11	11
State of nutrition.	Experimental day, fed	1–15	1–15	1–41	1–32	1–16		
	Experimental day, fasted	16–23	16–29	42–52	33–41	17–25	1–11	1–11
Left adrenal.	Weight, *mg*.	367.0	221.1	219.2	288.8	335.0	160.0	214.7
	Epinephrine content. *mg*.	0.382	0.222	0.337	0.316	0.358	0.169	0.243
	Epinephrine content. Per gm. of gland, *mg*.	1.04	1.00	1.54	1.09	1.07	1.06	1.13
Right adrenal.	Weight, *mg*.	304.1	188.4	176.2	312.0	297.9	126.8	185.7
	Epinephrine content. *mg*.	0.329	0.174	0.285	0.337	0.338	0.160	0.196
	Epinephrine content. Per gm. of gland, *mg*.	1.08	0.92	1.62	1.08	1.13	1.26	1.06

TABLE III.

Influence of Acute Epinephrine Intoxication upon the Epinephrine Content of the Adrenals.

Rabbit		I ♀	II ♀	III ♀
Body weight, *kg.*		1.76	2.08	2.16
Amount of epinephrine, injected subcutaneously, per kilo of initial body weight.		Dec. 22 2.0 mg. 2 p.m. 1.0 " 3 " 1.0 " 4 " 1.0 " 5 " 1.0 " 6 "	Dec. 26 1.0 mg. 9 a.m. 1.0 " 2 p.m. Dec. 27 1.0 mg. 9 a.m.	Jan. 22 1.0 mg. 9 a.m. Jan. 23 1.0 mg. 9 a.m. Jan. 24 1.0 mg. 9 a.m. 1.0 " 12 " 1.0 " 2.30 p.m. 1.0 " 3.30 " 1.0 " 4.30 " 1.0 " 5.30 " 1.0 " 6.30 " 1.0 " 7.30 " 1.0 " 8.30 "
Killed.		7 p.m., Dec. 22	2 p.m., Dec. 27	8.50 p.m., Jan. 24
Left adrenal.	Weight, *mg.*	200.0	291.8	241.0
	Epinephrine content. *mg.*	0.226	0.397	0.300
	Epinephrine content. Per gm. of gland, *mg.*	1.13	1.36	1.24
Right adrenal.	Weight, *mg.*	186.1	247.9	214.3
	Epinephrine content. *mg.*	0.183	0.306	0.238
	Epinephrine content. Per gm. of gland, *mg.*	0.98	1.23	1.11

the vasoconstrictive substance of the adrenals. Studying the adrenals of fasted animals histologically, Borberg[11] observed some decrease of the chromaffin substance. In order to note the effect of fasting itself, therefore, I examined also the epinephrine content of the adrenals of fasted rabbits. The results will be seen in Table IV. Rabbits I to V in this table are the same animals used in Paper I (Rabbits I to V in Tables II, III, and IV[35]).

From these tables, it will be seen that neither the weight nor the epinephrine content of the adrenals was materially changed by subcutaneous injection of epinephrine.

Epinephrine Content of the Adrenals and the Sugar Content of the Blood after Subcutaneous Injection of Diphtheria Toxin.

Elliott[18] reported that in diphtheria intoxication epinephrine of the adrenals is liberated mostly through the stimulation passing the splanchnic nerve, and the direct effect of the toxin is very slight. He considers that this is an effort made by the animal to combat the fall of the blood pressure and other changes due to the action of the poison elsewhere. His results in diphtheria intoxication are quite similar to those in morphine intoxication. A marked depletion of the epinephrine store of innervated adrenals as compared with the denervated companion gland in rabbits whose death was due to various kinds of infection was also reported by Stewart and Rogoff.[39] Determining the epinephrine content of the blood taken from the adrenal veins during conditions of low blood pressure and shock, Bedford[36] observed that increased quantities of epinephrine were forced into the blood, and explained this as a result of hyperactivity of the adrenal. In his experiments there is no statement as to whether these increased quantities of epinephrine were enough to induce any hyperglycemia. Studying the histological changes of the adrenals in diphtheria intoxication, Bogomolez[37] reported that the cortex showed marked degenerative changes, while the medulla showed little or no such alterations, and also that both the cortex and medulla indicated an increased secretory activity. His findings seem to be in harmony with the report of Elliott.[18]

If the hyperglycemia after administration of morphine or other toxic substances is really due to an excess of epinephrine, and the decreased epinephrine content of the adrenals found in these cases indicates a hypersecretion of epinephrine, and furthermore, if the decreased epinephrine content of the adrenals in diphtheria intoxication is not, for the most part, due to the toxic effect upon

[36] Bedford, E. A., *Am. J. Physiol.*, 1917, xliii, 235.

[37] Bogomolez, A., *Beitr. path. Anat. u. allg. Path.*, 1905, xxxviii, 510.

TABLE IV

Epinephrine Content of the Adrenals of Fasted Rabbits.

Rabbit		I ♀	II ♀	III ♀	IV ♀	V ♀	VI ♀	VII ♂
Body weight.	Beginning, *kg*	1.90	1.88	2.48	2.66	1.92	1.90	2.04
	End, *kg*	1.46	1.30	1.83	1.84	1.56	1.60	1.80
Duration of fasting, *days*		11	10	9	8	8	6	7*
Left adrenal.	Weight, *mg*	131.7	101.5	249.0	237.9	137.2	100.5	529.6
	Epinephrine content. *mg*	0.192	0.220	0.366	0.533	0.215	0.108	0.783
	Epinephrine content. Per gm. of gland, *mg*	1.46	2.17	1.47	2.24	1.57	1.07	1.48
Right adrenal.	Weight, *mg*	127.5	80.1	201.2	201.7	130.9	106.5	494.1
	Epinephrine content. *mg*	0.156	0.146	0.322	0.516	0.200	0.111	0.620
	Epinephrine content. Per gm. of gland, *mg*	1.22	1.82	1.60	2.56	1.53	1.04	1.25

* In the evening of the 6th fasting day, 50 gm. of litchi nuts were given.

the epinephrine-secreting cells, a diminution of epinephrine in the adrenals may run parallel with hyperglycemia in diphtheria intoxication, as well as after administration of morphine or other substances. No report concerning the hyperglycemia in diphtheria intoxication could be found in the literature available. In many infectious diseases with fever, hyperglycemia has been observed. Asphyxia is sometimes considered to be its cause. In a few experiments, I have examined the blood sugar content and the epinephrine content of the adrenals in diphtheria intoxication. Diphtheria toxin (Mulford Company, M.L.D. = 0.005 cc.) was injected subcutaneously into well nourished rabbits. The sugar content of the blood, obtained from an ear vein, was determined by the Lewis-Benedict method. Body temperature was measured in the vagina. In all the cases hemorrhagic edema at the site of the injection and hyperemia of the adrenals were observed. The results are shown in Table V.

From this table it will be seen that with the exception of one case, the epinephrine content of the adrenals was markedly decreased in diphtheria intoxication. In none of the cases, however, was hyperglycemia observed. These results, of course, do not exclude the possibility of an increase in quantity of epinephrine pouring into the circulation. An excess of epinephrine might not be large enough to induce hyperglycemia. At any rate, we are able to say that a decreased epinephrine content of the adrenals is not necessarily accompanied by hyperglycemia. This accords with Schur and Wiesel's findings[14] in strenuous muscle work. Deficient glycogen storage is not a sufficient explanation, as epinephrine can cause a marked hyperglycemia even in fasted animals.[35]

In connection with studies in diphtheria intoxication, I also determined the epinephrine content of the adrenals of rabbits, poisoned with guanidine hydrochloride. The poisoned animals were obtained from Dr. C. K. Watanabe. The results are summarized in Table VI.

From this table, it will be seen that the epinephrine content of the adrenals was sometimes decreased, sometimes normal. All the animals were submitted to frequent bleeding and were usually moribund when used for estimating the epinephrine content of the adrenals. According to Bedford, the low blood pressure

after bleeding can induce an increased liberation of epinephrine from the adrenals. In the present experiments, therefore, no clear statement can be made as to whether guanidine hydrochloride itself, or the manipulations made for other experimental purposes, caused a diminution of the epinephrine content of the adrenals.

Change of Weight and Epinephrine Content of the Adrenal after Removal of the Companion Gland.

Extirpating the left adrenal from young rabbits and examining the companion gland several months later, Stilling[38] noticed an abnormal increase in the size of the latter. This was also confirmed by Simmonds[39] in rabbit and guinea pig experiments. Wiesel[40] reported that in rats, accessory adrenals, showing structure similar to the cortex of the adrenals, were found in 50 per cent of the cases examined, and these accessory adrenals showed a compensatory hypertrophy after removal of one adrenal. In case of hypertrophy of the adrenal, participation of the cortex seems to be generally accepted. As to the hypertrophy of the medulla, opinions of the investigators vary. Elliott and Tuckett,[41] Stoerk and von Haberer[42] accept hypertrophy of the adrenal medulla under certain conditions. Vassale[43] and Borberg,[11] however, contest this. As to the epinephrine content of the adrenal after removal of the fellow gland, Battelli and Ornstein[29] found no increase, but rather a decrease. In similar experiments with guinea pigs, Borberg[11] could not find any sign indicating hypertrophy or hypersecretion of the medulla of the remaining adrenal. In rabbit experiments, however, Stewart and Rogoff[30] found some increase of the epinephrine content of the remaining adrenal.

Many conditions which modify the carbohydrate metabolism are considered to become effective through an intermediation of the adrenals. Cramer[44] attributed the decrease of the glycogen content of the liver of thyroid fed animals to hypersecretion of the adrenals, and on the other hand, Herring[45] reported an increase of the weight and epinephrine content of the adrenals

[38] Stilling, H., *Virchows Arch. path. Anat.*, 1889, cxviii, 569.

[39] Simmonds, M., *Virchows Arch. path. Anat.*, 1898, cliii, 138.

[40] Wiesel, J., *Centralbl. Physiol.*, 1898, xii, 780.

[41] Elliott, T. R., and Tuckett, I., *J. Physiol.*, 1906, xxxiv, 332.

[42] Stoerk, O., and von Haberer, H., *Arch. mik. Anat.*, 1908, lxxii, 481.

[43] Vassale, *Arch. ital. Biol.*, 1905, xliii, cited by Vincent, S., Internal Secretion and the Ductless Glands, New York and London, 1912, 151.

[44] Cramer, W., *J. Physiol.*, 1915–16, l, p. xxxviii.

[45] Herring, P. T., *Quart. J. Exp. Physiol.*, 1916, ix, 391; 1917, xi, 47.

TABLE V.

Epinephrine Content of the Adrenals and Sugar Content of the Blood after Subcutaneous Injection of Diphtheria Toxin.

Rabbit	I ♀	II ♀	III ♀	IV ♀
Body weight, *kg*	1.72	1.50	1.82	1.48
Diphtheria toxin, *cc.* (M. L. D. = 0.005 cc.)	Jan 29 0.075 10 a.m. 0.075 2 p.m. Jan. 30 0.15 10 a.m. 0.075 2 p.m. 0.075 3 "	Jan. 31 0.4 2 p.m.	Feb. 1 0.2 11 a.m.	Feb. 4 0.3 11 a.m.
Blood sugar content, *per cent*	Jan. 29 0.115 10 a.m. 0.122 9 p.m. Jan. 30 0.099 8 a.m. 0.086 5 p.m.	Jan. 31 0.100 1 p.m. 0.092 7 "	Feb. 1 0.111 10 a.m. 0.109 8 p.m. Feb. 2 0.102 11 a.m. 0.100 3 p.m. 0.126 9 "	Feb. 4 0.105 4 p.m. Feb. 5 0.084 2 p.m. 0.083 9 " Feb. 6 0.073 11 a.m.
Body temperature, °*C.*	Jan. 29 38.6 10 a.m. 38.6 2 p m. 39.7 5 " Jan. 30 38.6 10 a.m. 38.2 2 p.m. 37.8 3 "	Jan. 31 38.8 4 p.m. 38.2 9 "	Feb. 1 38.4 11 a.m. 39.7 8 p.m. Feb. 2 38.1 11 a.m. 37.1 9 p.m.	Feb. 4 38.6 11 a.m. 39.0 4 p.m. Feb. 5 40.1 9 a.m. 38.6 2 p.m. 38.1 9 " Feb. 6 37.5 11 a.m.

Killed		10 p.m., Jan. 30	10 p.m., Jan. 31	9 p.m., Feb. 2	11 a.m., Feb. 6
Left adrenal.	Weight, *mg.*	188.5	155.1	181.7	164.3
	Epinephrine content. *mg.*	0.162	0.133	0.251	0.079
	Epinephrine content. Per gm. of gland, *mg.*	0.86	0.86	1.38	0.48
Right adrenal.	Weight, *mg.*	177.5	136.0	184.3	128.6
	Epinephrine content. *mg.*	0.147	0.110	0.247	0.090
	Epinephrine content. Per gm. of gland, *mg.*	0.83	0.81	1.34	0.70

after thyroid feeding. The latter statement, however, could not be confirmed in my previous experiments.[23] Among the factors which induce the hyperactivity of the adrenal, the removal of one adrenal is probably one of the most effective. In order to see how the adrenals react to the demand for hyperactivity, I made some investigations as to the changes of the weight and epinephrine content of the adrenal after removal of the companion gland.

Method.—Two albino rats of the same sex and nearly the same body weight were paired off into one set. The right adrenal was removed from one rat under ether; the left one from the other. The operative technique was the same as described in the preceding paper.[46] It took about 5 minutes from the beginning of ether administration to the removal of the gland. The weight and epinephrine content of the two extirpated adrenals were determined together. After a certain interval, the animals were killed by bleeding and the remaining adrenals were examined for their weight and epinephrine content. As many investigators reported that even a prolonged ether anesthesia did not cause any marked depletion of the epinephrine content of the adrenals, the changes due to the very short duration of ether anesthesia in my rats seem to have been very slight, if existing at all. The results are shown in Table VII.

From these data it will be seen that notwithstanding a widely ranging individual variation of the weight of the adrenals, the removal of one adrenal caused a marked increase in the weight of the companion gland, especially in the female, much more than could be due to an increase of the body weight. The absolute epinephrine content of the adrenals was also increased by removal of the companion glands; this increase, however, being much less in proportion to that of the weight of the glands. The epinephrine content of the adrenal, calculated per gm. of the gland, however, was decreased by extirpation of the companion gland. These data show an interesting contrast to my previous results on the thyroid fed rats, where neither the weight nor the epinephrine content of the adrenals differed from those of the normal rats.[23] After removal of one adrenal, the medulla as well as the cortex

[46] Kuriyama, *J. Biol. Chem.*, 1918, xxxiv, 287.

TABLE VI.

Epinephrine Content of the Adrenals after Subcutaneous Injection of Guanidine Hydrochloride.

Rabbit		I ♀	II ♀	III ♀	IV ♀
Body weight, *kg.*		2.12	2.46	2.25	1.96
Amount of guanidine, per kilo of body weight, *gm.*		Dec. 22 0.2 8 a.m. Dec. 23 0.1 8 a.m.	Jan. 3 0.2 8 a.m. Jan. 4 0.2 12 a.m.	Jan. 5 0.2 9 a.m.	Jan. 5 0.2 8 a.m. Jan. 6 0.05 2 p.m.
Blood samples taken.		Dec. 22, twice. " 23 "	Jan. 3, twice. " 4, once.	Jan. 5, twice. " 6, once.	Jan. 5, twice. " 6, once.
Killed		3 p.m., Dec. 23	1 p.m., Jan. 4	10 a.m., Jan. 6	3 p.m., Jan. 6
Left adrenal.	Weight, *mg.*	436.7	389.1	506.0	231.8
	Epinephrine content. *mg.*	0.272	0.406	0.433	0.233
	Epinephrine content. Per gm. of gland, *mg.*	0.62	1.04	0.86	1.00
Right adrenal.	Weight, *mg.*	343.0	376.1	459.6	197.4
	Epinephrine content. *mg.*	0.238	0.433	0.397	0.197
	Epinephrine content. Per gm. of gland, *mg.*	0.69	1.15	0.86	1.00

TABLE VII.

*Change of Weight and Epinephrine Content of Adrenal after Removal of the Companion Gland.**

Group.	Sex.	Adrenals.	Body weight.		Adrenals.		Epinephrine.			Interval between two determinations of 1st and 2nd pairs.
			Rat.	Average.	Weight.	Weight per 100 gm. of average body weight.	In two adrenals.	Per gm. of adrenal.	Per 100 gm. of average body weight.	
			gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*days*
I	M.	1st pair.	(*a*) 249.4 (*b*) 232.4	240.9	56.6	23.5	0.117	2.07	0.049	29
		2nd "	(*a*) 249.2 (*b*) 233.1	241.2	54.9	22.8	0.129	2.35	0.054	
II	M.	1st pair.	(*a*) 238.4 (*b*) 241.3	239.9	32.8	13.7	0.078	2.38	0.033	25
		2nd "	(*a*) 245.1 (*b*) 229.5	237.3	43.3	18.3	0.083	1.92	0.035	
III	M.	1st pair.	(*a*) 179.6 (*b*) 198.8	189.2	28.5	15.1	0.049	1.72	0.026	26
		2nd "	(*a*) 185.3 (*b*) 240.5	212.9	37.6	17.7	0.087	2.31	0.041	
IV	M.	1st pair.	(*a*) 184.2 (*b*) 192.7	188.5	39.2	20.8	0.102	2.60	0.054	28
		2nd "	(*a*) 214.3 (*b*) 231.6	223.0	48.3	21.7	0.109	2.26	0.049	
V	M.	1st pair.	(*a*) 142.9 (*b*) 126.1	134.5	21.7	16.1	0.038	1.75	0.028	24
		2nd "	(*a*) 172.2 (*b*) 145.8	159.0	43.6	27.4	0.056	1.29	0.035	

VI	F.	1st pair.	(*a*) 267.2 (*b*) 256.2	261.7	52.6	20.1	0.135	2.57	0.052	30
		2nd "	(*a*) 288.3 (*b*) 294.6	291.5	75.9	26.0	0.183	2.41	0.063	
VII	F.	1st pair.	(*a*) 161.6 (*b*) 199.6	180.6	35.5	19.7	0.099	2.79	0.055	30
		2nd "	(*a*) 202.0 (*b*) 228.5	215.3	63.2	29.4	0.133	2.10	0.062	
VIII	F.	1st pair.	(*a*) 168.0 (*b*) 180.6	174.3	34.7	19.9	0.083	2.39	0.048	29
		2nd "	(*a*) 188.6 (*b*) 182.9	185.8	56.5	30.4	0.102	1.81	0.055	
IX	F.	1st pair.	(*a*) 152.7 (*b*) 152.9	152.8	38.7	25.3	0.074	1.91	0.048	24
		2nd "	(*a*) 178.5 (*b*) 184.2	181.4	54.2	29.9	0.100	1.85	0.055	
X	F.	1st pair.	(*a*) 128.5 (*b*) 115.5	122.0	26.1	21.4	0.045	1.72	0.037	24
		2nd "	(*a*) 151.9 (*b*) 130.1	141.0	43.0	30.5	0.078	1.81	0.055	
Average.		1st pair of adrenals.....		188.4	36.6	19.6	0.082	2.19	0.043	
		2nd " " "		208.8	52.1	25.4	0.106	2.01	0.050	
		Comparison, *per cent*†.		+10.8	+42.3	+29.6	+29.3	−8.2	+16.3	

* In each set in (*a*) the left adrenal was first removed; in (*b*) the right adrenal was first removed.

† Comparison of the first and second pairs of adrenals, employing the first pair of adrenals as a standard. The data are expressed in terms of percentages.

of the remaining adrenal may show a marked hypertrophy, but owing to an increased demand for epinephrine, its content in the medulla, in proportion to the weight of the latter, may be less than that in the normal adrenal medulla. Judging from the statements of many investigators, however, the adrenal medulla seems to be less likely to show hypertrophy than the cortex. The marked increase of the weight of the adrenal after removal of the companion gland in my experimental animals, is presumably mostly due to the increase of the size of the cortex, the medulla having much less participation. This may well explain a decrease of the epinephrine content of the enlarged adrenals, calculated per gm. of the gland. Generally it is not so easy to determine whether the secretory activity of the adrenal medulla is increased or not, merely from the epinephrine content of the adrenals. Some changes in the cortex must be taken into consideration. Furthermore, it is very probable that under certain conditions hyperactivity of the adrenal medulla can be accompanied by either an increased or a decreased storage of epinephrine.

SUMMARY.

The epinephrine content of the adrenals, extirpated without damage to their form and kept aseptically at room temperature and in air, decreases markedly within a few days. If the glands are kept in oxygen-free medium, the other conditions being the same, they do not lose their epinephrine store to any detectable degree.

The epinephrine content of the adrenals of rabbits is not materially changed by prolonged fasting.

The epinephrine content of the adrenals of rabbits is not materially changed either by acute epinephrine intoxication or by daily repetition of subcutaneous injection of epinephrine for a considerable length of time.

In diphtheria intoxication, the epinephrine content of the adrenals was usually decreased. The blood sugar content, however, was never increased. Parallelism of hyperglycemia and a decrease of the epinephrine content of the adrenals, reported by some investigators in asphyxia, *piqûre*, morphine, or carbon monoxide intoxication, could not be observed in diphtheria intoxication.

Notwithstanding a wide individual variation of the weight of the adrenals, the removal of one adrenal caused a marked increase in the weight of the companion gland, especially in the female, much more than could be due to an increase of the body weight. The absolute epinephrine content of the adrenals was also increased by removal of the companion gland; this increase, however, being proportionally much less than that of the weight of the glands. The epinephrine content of the adrenals, calculated per gm. of the gland, however, was decreased by extirpation of the companion gland.

It is difficult to state merely from the epinephrine content of the adrenals whether or not the secretory activity of the adrenal medulla is increased. Changes in the cortex and a possibility that under certain conditions hyperactivity of the adrenal medulla can be accompanied by either an increased or a decreased storage of epinephrine, must be taken into consideration.

The author desires to express his thanks to Professor Frank P. Underhill for his suggestions and interest throughout the course of this and the two preceding papers.

THE PHYSIOLOGICAL BEHAVIOR OF RAFFINOSE. II.

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Raffinose is widely distributed in the vegetable kingdom. It has been demonstrated in cotton seeds (1, 2, 3, 4), barley grains (5), wheat sprouts (6,7), eucalyptus manna (3,8), leaves and twigs of *Taxus baccata*(9), sugar beet (10,11,12), seeds of some leguminous plants (*Erythrina fusca, Entada scandens*) (13), and seeds of jute plants(14). The special enzyme splitting this trisaccharide has never been demonstrated in the alimentary tract of higher animals. In an earlier paper, Kuriyama and Mendel (15) observed that notwithstanding the absence of raffinase in the walls of the alimentary tract and glands discharging into it, raffinose can easily be split in the large intestine of rats and dogs. This was attributed mostly to bacterial action. In order to obtain further data concerning the physiological behavior of raffinose, I have made some experiments as to the raffinose-splitting power of extracts of feces, the presence of raffinase in some kinds of seeds, the fate of raffinase taken by mouth, and in a preliminary way regarding some properties of raffinase.

Some Properties of Raffinase, Compared with Those of Sucrase.

Enzymes which are active only with their coenzymes, can be made inactive very easily by removing the coenzymes by either dialysis or filtration through a porous clay filter. According to Onodera(16), the urease of soy bean loses its activity after dialysis. Umeda(17) reported that Rosenheim's coenzyme of pancreatic lipase could easily be separated by filtration through a Pukall porous clay filter. In my present experiments it was often neces-

sary to use dialysis or filtration through a clay filter in extracting the enzyme from feces or seeds. Destruction of enyzmes by heating is greatly modified by the character of the medium. An enzyme in a somewhat purified condition is more easily destroyed by heat. If the amount of the enzyme contained in the original material is very small, the enzyme extracted therefrom and placed in a more simple environment may be very easily destroyed even at the temperature usually employed for purposes of digestion, while the same amount of the enzyme might be well protected from destruction by heat and exert a strong activity in the original medium. As preliminary experiments, therefore, I have examined the influence of such manipulations upon the activity of yeast raffinase.

TABLE I.

Influence of Filtration through a Pukall Filter upon the Activity of Raffinase and Sucrase in the Same Preparation.

Experiment.	Enzyme.	Inversion of raffinose.		Inversion of sucrose.	
		Rotation.	Reduction.	Rotation.	Reduction.
		V.°		V.°	
I	Not filtered	+2.24	+++	−1.90	+++
	Filtered, I	+2.31	+++	−1.75	+++
	" II	+2.41	+++	−1.73	+++
	Boiled	+3.70	−	+4.70	−
II	Not filtered	+2.60	+++		
	Filtered, I	+2.88	+++		
	" II	+2.89	+++		
	Boiled	+3.70	−		

A solution of yeast raffinase was prepared from compressed yeast (Fleischmann's) as described in the first paper (15). It was neutral to litmus and contained 50 mg. of nitrogen per 100 cc. It showed a positive biuret reaction, but negative Fehling's test. As this enzyme solution contained both raffinase and sucrase, the influence of the experimental manipulations upon sucrase was also studied for comparison.

Influence of Filtration Through a Clay Filter.—The enzyme solution was filtered through a Pukall porous clay filter. Filtrates were obtained from two different filters. For testing the enzy-

matic activity of the solution, the mixture was made as follows: 3 cc. of 5 per cent raffinose (or 3 cc. of 10 per cent sucrose) + 5 cc. of enzyme solution + 10 drops of toluene. All the mixtures were incubated in stoppered bottles at 38°C. At the end of 2 hours, 1 cc. of 3 per cent $HgCl_2$ solution, 14 cc. of water, and 1 cc. of colloidal iron solution were added. The protein-free filtrate was examined polariscopically in a 2 dm. tube. The results are shown in Table I, from which it will be seen that the filtration of the solution containing both raffinase and sucrase through a clay filter induces little or no decrease in enzymatic activity.

Influence of Dialysis.—In preparing the original enzyme solution, it was subjected once to dialysis through parchment paper to make it neutral and free from reducing substances. To ascer-

TABLE II.

Influence of Dialysis upon the Activity of Raffinase and Sucrase in the Same Preparation.

Enzyme.	Inversion of raffinose.		Inversion of sucrose.	
	Rotation.	Reduction.	Rotation.	Reduction.
	V.°		V.°	
Not dialyzed	+2.75	+++	−0.10	+++
Dialyzed (parchment paper), I.	+2.62	+++	−0.20	+++
" " " II.	+2.59	+++		
Dialyzed (animal membrane), I.	+2.55	+++	−0.30	+++
" " " II.	+2.60	+++		
Boiled	+3.71	−	+4.69	−

tain whether further dialysis can alter the activity of the raffinase and sucrase, the following experiments were made: 10 cc. of the enzyme solution mixed with toluene were put in a sack of either parchment paper or an animal membrane, and immersed in 65 cc. of distilled water. The dialysis was continued for 3 days, the water being changed three times a day. The control sample was always kept in the same place. At the end of the dialysis, the volume of the enzyme solution was slightly increased. The control sample was therefore diluted in the same proportion. The activity of the enzyme solution was estimated as described in the previous experiments. The results are shown in Table II, which shows that dialysis did not materially affect the activity of either raffinase or sucrase.

Influence of Heat.—The enzyme solution, mixed with a small amount of toluene and kept in a stoppered bottle, was incubated at 38–40°C., the control sample being kept at room temperature. The results of a subsequent test are detailed in Table III, which shows that a small amount of raffinase in a rather simple medium is easily destroyed at the temperature of 38–40°C. Prolonged incubation is therefore of no advantage in experiments of this sort.

TABLE III.

Influence of Temperature of 38–40°C. upon the Activity of Raffinase and Sucrase in the Same Preparation

Experiment No.	Enzyme, heat application.	Inversion of raffinose.		Inversion of sucrose.	
		Rotation.	Reduction.	Rotation.	Reduction.
		V.°		V.°	
I	At room temperature for 3 days	+2.75	+++	−0.10	+++
	" 38–40°C. for 3 days, I	+3.68	−	+4.65	−
	" 38–40° " " 3 " II	+3.71	−	+4.65	−
II	At room temperature for 2 days	+2.65	+++	−0.15	+++
	" 38–40°C. for 2 days	+3.70	−	+4.65	−
III	At room temperature for 1 day	+2.20	+++	−1.31	+++
	" 38–40°C. for 1 day, I	+2.35	+++	−1.32	+++
	" 38–40° " " 1 " II	+2.30	+++	−1.25	+++
IV	At room temperature for 20 hrs.	+2.29	+++	−1.05	+++
	" 38–40°C. for 20 hrs., I	+2.48	+++	−0.85	+++
	" 38–40° " " 20 " II	+2.50	+++	−0.87	+++

Presence of Raffinase in the Feces.

The presence of raffinase in bacteria, yeasts, and molds has been mentioned by many investigators (18, 19, 20, 21). Winslow (22) reported that the bacillus of the colon group which can utilize the ketonic disaccharide can always utilize the ketonic trisaccharide, raffinose, as well. Testing the raffinose-attacking power of fourteen strains of *Bacillus coli* and two strains of *Bacillus acidophilus*, obtained from the Bacteriological Laboratory of the Yale School of Medicine and the Sheffield Laboratory of Bacteriology,

I found that none of the strains of *Bacillus coli* had ability to attack raffinose, while both strains of *Bacillus acidophilus* possessed this power. Pringsheim and Zemplén (23) reported that the press juices of various kinds of fungi contained a raffinose-splitting enzyme, the carbohydrates being sometimes split into sucrose and galactose, at other times into melibiose and fructose. I could also demonstrate a considerable amount of raffinase in the commercial taka-diastase, which is said to be manufactured from *Aspergillus oryzæ*. According to Anderson (24), many types of yeast-like fungi, for the most part such types as are commonly present in nature and known as wild yeasts, are found in the intestinal tract of man. He considers that they are ingested with the food. As factors which split raffinose in the alimentary canal, the living microorganisms, including bacteria, yeasts, and molds, and the enzyme isolated from these organisms, must be taken into consideration. The presence of a small amount of raffinase in the sterile extract of the feces of dogs and rabbits has been reported in an earlier paper (15). Further experiments have now been made.

Methods.—The fresh feces of man, calf, pig, dog, rabbit, and guinea pig were ground with water and made to a 10 per cent suspension. This was kept at room temperature for 4 to 96 hours, and shaken from time to time. Toluene was used as an antiseptic. The suspension was later filtered through a Buchner funnel and the filtrate then drawn through a sterilized Pukall filter. The final clear sterile filtrate, reacting slightly acid to litmus, and containing no reducing substance, was tested for its raffinose-splitting power. The sterility of the feces extract was tested with agar-agar culture media. The digestion mixture was made as follows: 10 cc. of feces extract + 3 cc. of 5 per cent raffinose + 10 drops of toluene. After incubating this mixture for 20 to 40 hours at 38–40°C., 15 cc. of water and 2 cc. of colloidal iron solution were added. The protein-free filtrate was examined for its reducing power by Benedict's test. For control experiments, the boiled feces extract was used. If the whole amount of raffinose is decomposed into melibiose and fructose or into glucose, galactose, and fructose, the rotatory power of the testing material decreases respectively to one-half or one-fifth of the original, the change of the rotatory power, therefore, being much less than

in the case of sucrose. In the mixture of the feces extract and raffinose, the decrease of the rotatory power was usually very slight, and sometimes rather uncertain, while the appearance of the reducing power clearly indicated the decomposition of a certain amount of raffinose. In the present experiments, therefore, estimation of the reducing power only was made. The results are shown in Table IV, from which it will be seen that the sterile extract of the feces usually contained a small amount of raffinase. Change of the diet, in rabbit experiments, did not alter the raffinose-splitting power of the feces extract.

TABLE IV.

Raffinose-Splitting Power of the Feces Extract.

Source of material.	Food.	Solid substance of feces.	Duration of extraction.	Duration of incubation.	Reduction.	
					Original sample.	Boiled sample.
		per cent	*hrs.*	*hrs.*		
Man	Mixed diet.	22.3	4	20	+	—
Calf I.		23.1	4	20	—	—
			48	20	—	—
" II.		22.1	4	20	—	—
			48	40	±	—
Pig.		28.8	4	40	+	—
			48	40	+	—
Dog I.	Meat.	56.9	4	40	+	—
" II.	"	59.1	4	40	—	—
			48	40	—	—
Rabbit I.	Oats.	88.4	4	40	+	—
" II.	Cabbage.	52.1	48	40	+	—
Guinea pig, Group I.	Oats + cabbage.	51.8	4	40	+	—
" " " II.	Oats + cabbage.	51.2	96	40	+	—

In order to find out whether the feces extract prepared as mentioned above was favorable for the activity of raffinase or not, the activity of yeast raffinase was determined in the medium of the feces extract. The extracts were slightly acid. The procedure was generally the same as described before, the mixture of the enzyme and substrate being incubated for 2 hours. The results are detailed in Table V, which shows that the extract of the feces of rabbits and guinea pigs markedly decreased the activity of yeast raffinase.

TABLE V.

Influence of Feces Extract upon the Activity of Yeast Raffinase.

Mixture: raffinose plus		Inversion of raffinose.	
Enzyme.	Feces extract or water.	Rotation.	Reduction.
		V.°	
Enzyme	Water.	+2.10	+++
"	Extract of rabbit feces.	+2.85	++
"	" " " "	+2.90	++
"	" " guinea pig feces.	+3.30	++
"	" " " " "	+3.33	++
Boiled enzyme	Water.	+3.70	−

Presence of Raffinase in a Few Kinds of Seeds.

Raffinase has been found in various kinds of plants; in the root of the beet, in germinating barley and wheat, and elsewhere (25). Bailey demonstrated raffinase in bananas (26). In the present experiments I have examined mung bean (*Phaseolus aureus*), mung bean sprout, cotton seed, and soy bean for the presence of raffinase.

Methods.—In the case of seeds, the material was first powdered and ground with an addition of five times its weight of water and a certain amount of sand. The suspension was kept with toluene at room temperature for 3 hours, and shaken from time to time. It was then filtered on a Buchner funnel, and the filtrate was dialyzed for 2 days through parchment. Toluene was used as an antiseptic. The precipitate that occurred during dialysis was filtered off. The filtrate was neutral and showed neither Fehling's reaction nor a starch reaction with iodine. The procedure for

estimating the raffinose-splitting power was the same as described before. Duration of incubation was 48 hours. For determination of raffinase in mung bean sprouts, the seeds, soaked in water, were kept in a sunny place. The sprouts, from 5 to 7 days old, were ground together with the same weight of water and a small amount of sand. The further manipulations were the same as in the case of seeds. The results are shown in Table VI, in which it will be seen that mung bean, its sprouts, cotton seed, and soy bean contained a small amount of raffinase.

TABLE VI.

Raffinase in a Few Kinds of Seeds.

Material.	Sample No.	Inversion of raffinose.			
		Original sample.		Boiled sample.	
		Rotation.	Reduction.	Rotation.	Reduction.
		V.°		V.°	
Mung bean	I	+3.60	+	+3.70	−
	II	+3.40	+	+3.70	−
Mung bean sprout	I 5 days old	+3.30	+	+3.68	−
	II 6 days old	+3.11	+	+3.70	−
	III 7 days old	+3.30	+	+3.69	−
Cotton seed	I	+2.88	+	+3.68	−
	II	+2.93	+	+3.71	−
	III	+3.41	+	+3.70	−
	IV	+2.25	++	+3.70	−
Soy bean	I	+3.05	+	+3.72	−

Fate of Yeast Raffinase in the Alimentary Tract.

Enzymes taken by mouth are usually, for the most part, destroyed in the stomach. If they are well protected by other food substances, however, part of them can reach the intestine in active condition. Neumayer (27) reported that some kinds of yeast can pass through the entire alimentary canal of man and animals (rabbits and cats) without entirely losing their fer-

menting power. Kudo (28) demonstrated that administration of yeast or yeast preparations by mouth somewhat increases the fermenting power of the intestinal contents, the maximum being reached within a few hours. On feeding various kinds of yeast to a healthy man, Anderson (24) noticed that the yeasts can pass through the whole alimentary canal in active condition. Long and Hull (29) found that notwithstanding an abundant secretion of pepsin and hydrochloric acid, trypsin was not destroyed in the stomach, provided sufficient protein was present to bring the concentration of the free acid down to a certain value. In a few experiments I have examined whether yeast raffinase fed to dogs and rabbits can reach the intestine without being entirely destroyed in the stomach.

Rabbit Experiments.—10 gm. of compressed yeast (Fleischmann) made as a 20 per cent suspension were given by a stomach sound daily to two rabbits for 4 successive days. The animals were fed on oats, water being given freely. The feces of the 3rd and 4th experimental days were collected together and also that of the 5th and 6th days. They were examined for the presence of raffinase by the procedure previously described. The raffinose-splitting power of the feces of these animals, while taking their ordinary food, was also estimated for comparison. The results are shown in Table VII. From the data in this table, it will be seen that the raffinose-splitting power of the feces was increased by yeast feeding.

Dog Experiments.—A suspension of compressed yeast was given by sound into the empty stomach or 20 minutes after meat feeding. After yeast feeding the secretion of the gastric juice was facilitated by sham feeding—placing a piece of meat in front of the cage. 1 to 2 hours later, the stomach contents were aspirated and tested for the total acidity, free hydrochloric acid, and the raffinose-splitting power. For estimating the latter, the filtrate of the stomach contents was first neutralized by a sodium hydroxide solution. In the final experiment with the same dog, the animal was killed 1½ hours after yeast feeding and the contents of the stomach, duodenum, and jejunum were examined. The results are shown in Table VIII, A.

Experiments with Artificial Gastric Juice.—5.0 gm. of compressed yeast were ground together with 50 cc. of an artificial

gastric juice (0.2 per cent hydrochloric acid and 0.3 per cent scale pepsin) and 20 drops of toluene and incubated at 38°C. for 1 to 4 hours. At the end of the desired length of time, 5 cc. of the homogeneous suspension were neutralized with NaOH and kept in an ice box. As control experiments, yeast suspension in the same artificial gastric juice was immediately neutralized and kept in an ice box. As a further control, the yeast suspension was immediately neutralized and boiled. When the specimen incubated for 4 hours was neutralized and ready for further manipulations, all the specimens, including the control specimens and

TABLE VII.

Raffinose-Splitting Power of the Feces Extract after Yeast Feeding.

Rabbit.		Diet.	Date of sample.	Solid substance of feces.	Inversion of raffinose			
					Original sample.		Boiled sample.	
No.	Body weight.				Rotation.	Reduction.	Rotation.	Reduction.
	kg.			*per cent*	*V.°*		*V.°*	
		Oats.		88.4	+2.79	+	+2.93	−
I	2.62	Oats. Addition of yeast, June 7–10.	June 9, 10	82.0	+2.48	++	+2.90	−
			" 11, 12	83.2	+2.30	++	+2.90	−
		Cabbage.		52.1	+2.75	+	+2.91	−
II	2.24	Oats. Addition of yeast, June 7–10.	June 9, 10	85.7	+2.38	++	+2.93	−
			" 11, 12	82.0	+2.51	++	+2.90	−

those kept in the ice box after incubation, were examined together for their raffinose-splitting power. The results are shown in Table VIII, B. From these data it will be seen that yeast raffinase is, for the most part, destroyed by the gastric juice. In suitable conditions, however, part of it can reach the intestine in active condition. In discussing the raffinose-splitting power in the intestine the part of the raffinase taken with the food, which has escaped from being destroyed in the stomach, must be taken into consideration.

TABLE VIII.

Influence of Gastric Digestion upon Yeast Raffinase.

A. Experiments with a Dog (♀, 7.4 Kg.).

Experiment				I	II	III	IV
Food.	Meat, *gm.*			50	30		
	Compressed yeast, *gm.*			15	15	20	20
	Water, *cc.*			150	250	250	250
Time of aspiration of stomach content after feeding, *hrs.*				1	1	1	2*
Stomach content.	Total acidity			90	26	15	Neutral.
	Free HCl			50	12.5	11	
	Raffinose-splitting power.	Original sample.	Rotation, V.°	+2.00	+1.95	+1.95	+3.65
			Reduction.	+++	+++	+++	±
		Boiled sample.	Rotation, V.°	+3.65	+3.68	+3.70	+3.67
			Reduction.	−	−	−	−

Experiment V.—Food: Meat, *gm.* 50; Compressed yeast, *gm.* 15; Water, *cc.* 150

1½ hrs. after feeding, the animal was killed with chloroform.

Contents of alimentary tract.

			Stomach.	Duodenum.	Jejunum (60 cm. from beginning).
Weight of content, *gm.*			63.8	6.9	18.7
Reaction			Total acidity, 65. Free HCl, 25.	Slightly acid.	Slightly acid.
Raffinose-splitting power.	Original sample.	Rotation, V.°	+1.80	+3.15	+3.01
		Reduction	+++	+	+
	Boiled sample.	Rotation, V.°	+3.70	+3.68	+3.70
		Reduction	−	−	−

* The stomach being empty, it was washed with 50 cc. of water.

TABLE VIII—*Concluded.*

B. Experiments with Artificial Gastric Juice.

Duration of treatment with gastric juice.	Experiment I. Rotation.	Experiment II. Rotation.
hrs.	V.°	V.°
0	+4.13	+4.08
½	+5.20	+5.25
1	+6.15	+6.12
2	+6.35	+6.39
3	+6.35	+6.35
4	+6.32	+6.35
Control (boiled enzyme).	+6.35	+6.35

SUMMARY.

The activity of yeast raffinase is not materially decreased by filtration through a clay filter or by dialysis.

The sterile extract of feces usually contains a small amount of raffinase. The extract of feces markedly decreases the activity of yeast raffinase.

Mung bean, its sprouts, cotton seed, and soy bean contain a small amount of raffinase.

Yeast raffinase, taken by mouth is, for the most part, destroyed by the gastric juice. Under suitable conditions, however, part of it can reach the intestine in active condition. The raffinose-splitting power of the sterile extract of feces can be increased by yeast feeding.

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BLOOD DEXTROSE AS AFFECTED BY MORPHINE AND MORPHINE WITH ETHER ANESTHESIA.

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For many years morphine has been used by surgeons introductory to other anesthesia. It reduces the excitement stage of anesthesia and lessens the amount of ether necessary to maintain anesthesia (1).

The amount of dextrose in the blood is influenced by many factors. Allen (2) states that special agencies described as increasing the blood sugar are: muscular labor, heat, cold, hemorrhage, traumatism, nervous or emotional disturbances, drugs and infectious and other diseases. Asphyxia is said to cause hyperglycemia. It would be difficult to find a factor in the body affected by such a variety of agents as the blood sugar. The range of variation is considerable, amounting to the tripling of the normal value in many cases (3). Thus the author is inclined to consider the measure of the blood dextrose changes a rough measure of the shock sustained by a living body as the result of the application of various agents to that body.

The point attacked in this work is whether morphine increases or decreases the unbalanced physiological condition produced by ether anesthesia. The variations in blood dextrose are taken as the indicator.

EXPERIMENTAL.

Blood dextrose was determined according to the modification of the Lewis-Benedict colorimetric method used by Myers and Bailey (4). Blood was taken from the external jugular into a syringe containing powdered sodium oxalate. Sufficient quantities were taken to make triplicate determinations.

The animals were healthy dogs which had not been prepared in any way for the work, except that they were never fed within 18 hours of the time the experiment began. Three groups of dogs were used.

The first group of animals consisted of eleven dogs and each dog was used once. They were bled and immediately given, hypodermically, 0.25 cc. of a 4 per cent solution of morphine per kilo of body weight. This amounted to 0.01 gm. of morphine sulfate per kilo of body weight, and was used in accordance with the dosage as a narcotic for dogs recommended by Sollmann (5). Half an hour after giving the morphine the dogs were bled again. 15 minutes later they were bled. After 45 minutes more the final sample was taken. The averages of the determinations made in each case are given in Table I.

The second series of dogs consisted of eight animals and three of these were used twice each. These animals were given the same dose of morphine as the first set of dogs. Half an hour after giving the morphine they were bled, and then immediately anesthetized. Ether was administered as a vapor driven into a cone by an air blast passing through a bottle of ether. This was for the purpose of furnishing plenty of oxygen to the animal. After the animal had been under the anesthetic 15 minutes, a second sample was taken. 45 minutes later while the animal was still anesthetized a final blood sample was taken. The results obtained are given in Table II.

The third series consisted of ten dogs. They were bled and immediately anesthetized. The method of anesthetization was the same as in the second group. After 15 minutes of anesthesia the second blood was taken. Immediately the usual dose of morphine was injected. Half an hour later the final sample of blood was taken. The results of the analyses are given in Table III.

DISCUSSION.

From Table I it is found that the dextrose content of the blood of normal dogs varied from 0.082 to 0.154 per cent. The eleven dogs averaged 0.1186 per cent. After morphine had acted half an hour, the blood dextrose varied from 0.109 to 0.275 per cent, averaging for the eleven dogs 0.1883 per cent. In every case

the blood sugar was increased. Calculating from the averages, half an hour's action of morphine increased the dextrose content 59 per cent of the original value. 15 minutes later or 45 minutes' action of morphine caused the values to vary from 0.120 to 0.331 per cent, averaging 0.1966 per cent. Compared with the values 15 minutes earlier there were seven increases, two non-appreciable drops, and two considerable decreases. Calculating from the averages it is found that 45 minutes' action of morphine increased the blood sugar 66 per cent of the original value. After morphine had acted an hour and a half, the dextrose of the blood

TABLE I.

Effect of Morphine on Blood Sugar.

Dog.	Weight of dog.	Amount of morphine.	Dextrose.			
			Before morphine.	½ hr. after morphine.	¾ hr. after morphine.	1½ hrs. after morphine.
	kg.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
1	10	0.10	0.090	0.109	0.120	0.102
2	11	0.11	0.120	0.180	0.154	0.178
3	10	0.10	0.137	0.226	0.220	0.233
4	11	0.11	0.142	0.171	0.181	0.162
5	10	0.10	0.154	0.184	0.146	0.255
6	16	0.16	0.121	0.263	0.276	0.247
7	10	0.10	0.127	0.166	0.180	0.206
8	10	0.10	0.082	0.142	0.184	0.248
9	16	0.16	0.111	0.275	0.331	0.307
10	10	0.10	0.115	0.209	0.207	0.191
11	15	0.15	0.106	0.146	0.164	0.175
Average			0.1186	0.1883	0.1966	0.2095

varied from 0.102 to 0.307 per cent, averaging 0.2095 per cent. Five of the eleven dogs did not show an increase after the end of the first 45 minutes. However, the average value shows an increase of 77 per cent of the blood dextrose before morphine. From the figures obtained it is clear that morphine brings on a pronounced hyperglycemia and the major part is accomplished in the first half hour. These results are what would be expected in the light of the work done by Luzzatto and Araki. Scott (6) states that Luzzatto obtained glycosuria in rabbits and Araki in dogs as the result of the administration of morphine.

In Table II the blood sugar is evaluated during the action of morphine, before and after ether has been given. After half an hour's action of morphine the blood dextrose varied from 0.118 to 0.236, averaging 0.1725 per cent dextrose. In Table I the average value after half an hour's action of morphine was 0.1883 to 0.0157 per cent higher. 15 minutes of ether anesthesia increased the hyperglycemia in every case, averaging 0.2046 per cent. This increase amounts to 19 per cent of the value before ether and after half an hour's action of morphine. The increase due to this 15 minutes' action of morphine, calculated from the data in Table I was 4 per cent of the value after half an hour's action of morphine. Thus it would appear that ether was capable of raising the blood sugar 15 per cent in 15 minutes when acting with morphine. However, it is interesting to note that the blood sugar after 45 minutes' action of morphine and 15 minutes' action of ether is 0.2046 per cent, while after 45 minutes' action of morphine alone it is 0.1966 per cent, only 0.0080 per cent difference in favor of the ether.

After the animals had been under ether 1 hour and still under the influence of the morphine, the average dextrose content was 0.2085 per cent. This is an increase of 21 per cent of the content before ether. This increase of 21 per cent is due to the influence of 1 hour of ether anesthesia and the last two half hours of an hour and a half's action of morphine. Calculating from the data of Table I the influence during the last hour of an hour and a half's action of morphine alone amounted to 11 per cent of the value at the end of the first half hour. This leaves an increase of only 10 per cent due to the ether itself in the whole 1 hour of ether anesthesia. It is interesting to note that with the action of morphine alone for an hour and a half the glycemia is 0.2095, while with the added influence of an hour of ether anesthesia, the glycemia is 0.2085—almost identical.

The data given in Table III give first the blood dextrose of ten normal dogs, second the blood sugar after 15 minutes of ether anesthesia, and third the same after half an hour's further action of ether anesthesia and the added influence of morphine. The variation of the normal blood sugar in this series of dogs was from 0.096 to 0.149 per cent as against 0.082 to 0.154 per cent in the series given in Table I. The average in this series amounted

to 0.1101 per cent, while that of the first series was 0.1186 per cent. Calculating the average normal glycemia in the twenty-one dogs included in the two series, it is found to amount to 0.1145 per cent of dextrose.

The action of 15 minutes of ether anesthesia increased every glycemia. The results varied from 0.132 to 0.277, averaging 0.1784 per cent. The increase due to the ether was 62 per cent of the normal.

TABLE II.

Effect of Ether after Morphine on Blood Sugar.

Dog.	Weight of dog.	Amount of morphine.	Dextrose.		
			Before ether, ½ hr. after morphine.	After 15 min. ether anesthesia.	After 1 hr. ether anesthesia.
	kg.	*gm.*	*per cent*	*per cent*	*per cent*
1	10	0.10	0.145	0.162	0.176
1	10	0.10	0.220	0.230	0.265
2	10	0.10	0.168	0.222	0.213
2	10	0.10	0.168	0.212	0.230
3	8	0.08	0.224	0.252	0.244
4	10	0.10	0.165	0.192	0.205
4	10	0.10	0.140	0.190	0.229
5	8	0.08	0.118	0.137	0.155
6	9	0.09	0.160	0.185	0.162
7	8	0.08	0.236	0.263	
8	8	0.08	0.154	0.206	0.206
Average			0.1725	0.2046	0.2085

The half hour's action of morphine during the anesthesia and following 15 minutes of anesthesia gave glycemias varying from 0.126 to 0.307, averaging 0.2110 per cent. Seven out of ten of the animals increased their amount of blood sugar. The average increase for all the animals of the series amounted to 0.0328 per cent of dextrose, or 18 per cent of the first value. Morphine alone in half an hour caused an increase of 0.0697 per cent of dextrose, or 59 per cent of the first value. This indicates that morphine does not produce such an increase in blood sugar when acting with ether as when acting alone. It is interesting to note that the final values in the two cases were 0.2110 and 0.2095 per cent respectively. They compare very closely.

It was thought that possibly the amount of blood taken as a sample might in itself cause an increase in blood sugar. Three dogs, exactly like those of the three series, were taken and bled 20 cc., each 15 minutes apart. Their blood sugars at the first and second bleedings were 0.138, 0.174, 0.151, and 0.138, 0.178, 0.145 respectively. The averages of the first and second bleedings were 0.1543 and 0.1537.

The results discussed are tabulated in Table IV.

In this table there are three large blood sugar increases. They are the results of (1) the action of morphine alone for half an

TABLE III.

Blood Dextrose Affected by Ether Anesthesia and Morphine after Ether Anesthesia.

Dog.	Weight of dog.	Amount of morphine.	Dextrose.		
			Before ether.	After 15 min. ether anesthesia.	After 45 min. ether and 30 min. morphine.
	kg.	*gm.*	*per cent*	*per cent*	*per cent*
1	8.4	0.084	0.105	0.168	0.248
2	13.0	0.130	0.120	0.250	0.205
3	8.4	0.084	0.149	0.277	0.307
4	8.2	0.082	0.107	0.172	0.148
5	11.8	0.118	0.113	0.152	0.205
6	10.0	0.100	0.101	0.213	0.234
7	8.2	0.082	0.100	0.137	0.226
8	17.0	0.170	0.096	0.132	0.125
9	10.0	0.100	0.103	0.132	0.215
10	7.3	0.073	0.107	0.151	0.196
Average			0.1101	0.1784	0.2110

hour, (2) the action of ether alone for 15 minutes, and (3) the action of ether alone for 1 hour. The increase in blood sugar due to the action of morphine for half an hour during ether anesthesia was less than without ether. The increase in blood sugar resulting either from 15 minutes or 1 hour's action of ether anesthesia after the administration of morphine was much less than without morphine.

From an inspection of the averages in Tables I, II, and III it appears that there is a maximum glycemia in the vicinity of

0.210 per cent beyond which there is no increase. This maximum glycemia seems to have been approximated in the case of (1) the action of morphine for an hour and a half, (2) the action due to 45 minutes of morphine and 15 minutes of ether, and (3) the action of morphine for an hour and a half with the addition of ether for 1¼ hours, the action of ether for 45 minutes and morphine for 30 minutes.

The small changes in the blood sugar recorded in Table IV will be observed to be from hyperglycemias near the limit. These differences are not considered of any significance in determining

TABLE IV.

Averages from Other Tables.

	Dextrose.			Per cent of difference (approximate).
	Before.	After.	Difference.	
	per cent	*per cent*	*per cent*	*per cent*
½ hr. morphine alone	0.1186	0.1883	0.0697	59
½ " " during ether	0.1784	0.2110	0.0326	18
15 min. ether alone	0.1101	0.1784	0.0683	62
15 " " during morphine	0.1725	0.2046	0.0321	19
15 " morphine (corresponding)	0.1883	0.1967	0.0084	4
1 hr. morphine after ½ hr. morphine	0.1883	0.2095	0.0212	11
1 " " and ether, after ½ hr. morphine	0.1725	0.2085	0.0360	21
1 hr. ether alone	0.0750*	0.1300*	0.0550	73

* Ross, E. L., and McGuigan, H., *J. Biol. Chem.*, 1915, xxii, 407.

the difference in the action of the two drugs. The limit of the animal to react is reached before the action of the drug is completed. The glycosuria found in animals by Luzzatto and Araki as a result of morphine administration indicates the reason for the limit to the hyperglycemia. When the glycemia is near the maximum glycemia, a hyperglycemia-producing drug can only raise the blood sugar to the limit even though the drug were capable of raising the blood sugar much more in a normal animal.

SUMMARY AND CONCLUSIONS.

A series of animals was subjected to the action of morphine injected subcutaneously and ether administered in an air mixture.

These drugs were applied alone and together and in different orders. Blood dextrose determinations were made at various intervals.

The data lead to the following conclusions:

1. Morphine administered hypodermically in dogs in doses of 10 mg. per kilo of animal produced an average increase of 59 per cent in the blood sugar in half an hour, 66 per cent in 45 minutes, and 77 per cent in an hour and a half.

2. Ether given after morphine has been acting for half an hour, does not cause as much increase in blood sugar as if the morphine had not been used. However, the final degree of hyperglycemia is approximately the same, either with or without morphine.

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DETERMINATION OF VARIOUS FORMS OF NITROGEN IN BOVINE FLESH, INCLUDING THE PRODUCTS OF HYDROLYSIS OF SOME OF THE PROTEINS.*

FIRST PAPER.

THE HEXONE BASES OF SOME FLESH PROTEINS.

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(Received for publication, March 3, 1918.)

Hausmann's (1) method for determining the nitrogen distribution into protein analysis and its subsequent modifications (2, 3, 4, 5, 6) has been widely used in the analysis of various proteins, tissues, foods, and feedingstuffs.

For the present study a number of beef proteins were analyzed according to the Van Slyke method. The proteins used represent the fractions of the flesh as described by Grindley (7). The cold water-insoluble protein is a mixture of stroma and plasma proteins and contains albuminoids and nucleoproteins. The cold water-soluble, heat-coagulable fraction is a mixture of plasma protein, the albumins and globulins of flesh.

Preparation of Samples.

The flesh was separated from the fatty tissue and bones by hand. It was then ground finely in a meat chopper and about 5 kilos were placed in a cooler the temperature of which was about 40°F., and mixed thoroughly with cold water. The mixture was then filtered through muslin. If the temperature is not too low labor can be saved by pouring the mixture upon the muslin which has been fastened on a square frame and catching the filtrate by means of a funnel in a bottle below. The mixing with water and

* This and the following paper represent part of the work incorporated in a thesis submitted by W. E. Thrun in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Agricultural Chemistry of the University of Missouri.

filtering is repeated until the filtrate no longer gives the biuret reaction. The filtrate is treated with a little thymol to prevent bacterial action and filtered through paper and evaporated on the water bath until quite concentrated. The coagulated proteins are filtered off[1] by means of suction and washed with water and finally with alcohol. The cold water-insoluble residue and the coagulable protein are mixed thoroughly with alcohol and preserved that way until used. The filtrate and aqueous washings from the coagulable protein are concentrated on the water bath to a thick syrup and preserved as such. The concentration is such that bacteria will not grow.

The cold water-insoluble residue is treated with alcohol and filtered by suction. This treatment is repeated several times. Then it is treated with ether and filtered several times. The alcoholic and ether filtrates are mixed and made up to volume, and the nitrogen is determined in an aliquot. The protein residue is air-dried, weighed, and the nitrogen determined in a sample. 10 gm. samples are placed in paper cones and extracted in a Soxhlet apparatus. Absolute alcohol is poured upon it first and it is allowed to stand for a few days. The alcohol is then siphoned off into the flask and the sample is further extracted with ether in the usual way for about 24 hours. The alcohol-ether mixture is washed into a Kjeldahl flask and the nitrogen in it determined in the usual way. The quantity of nitrogen obtained in the second extraction is very small. The sum of the weights of the nitrogen in the alcohol-ether extracts and the nitrogen in the residue after extraction divided by the weight of the nitrogen obtained by the two extractions gives the lipoid-protein nitrogen ratio of the cold water-insoluble part of the flesh. The extracted samples are then ready for analysis.

Van Slyke (8) found by determining the amino nitrogen that digestion of muscle proteins in water at 100°C. for 3 hours did not hydrolyze them. The water extracts of these samples were made in the cold so that enzymes present did not have favorable conditions during that time.

Description of Samples.

The cold water-insoluble protein, No. 562C, was obtained from a lean flesh composite of a new born calf, and No. 549 from a 5 year old stunted steer in the manner described above. Sample 508B was prepared as follows: The lean flesh of round of baby beef was extracted with cold water and then with ammonium sulfate, the remaining ammonium sulfate was removed by washing and the sample was treated with alcohol, dried, and then extracted with ether. The sample was then air-dried. The whitish shreds resulting were sifted and the coarsest sample was 508B.

The coagulable samples were obtained as described above, 562C from the lean flesh composites of a new born calf, and the others from the lean of the round. Steer 505 was full fed from birth and slaughtered at 11 months;

[1] If filtering goes slowly it is best to do it in the cold.

No. 501 was treated as No. 505 but slaughtered at 48 months; No. 48 was held as a feeder for 3½ years and then full fed for 1½ years. Nos. 505, 501, and 48 were all fat when slaughtered and analysis of the proteins from the same parts of the carcass ought to show the influence of age upon the composition of the sample. In this paper the cold water-insoluble residues are designated with an *R* before the number of the animal from which they came, the coagulated samples by *C*.

In Table I the results of the Van Slyke analysis are given.

TABLE I.

Van Slyke Analysis of Seven Beef Flesh Proteins in Per Cent of Total Nitrogen.

Sample.	Nitrogen.								
	Humin.	Ammonia.	Cystine.	Histidine.	Lysine.	Arginine.	Non-amino, in filtrate.	Amino, in filtrate.	Total.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
R 549. Steer, 5 yrs. Residue of lean of ribs, water extraction followed by alcohol and ether extraction.....	1.4	3.9	1.1	1.9 32.3	14.0	15.3	0.9 59.2	58.3	96.8
R 508B. Baby beef. Sample lean meat extracted with cold water, 10 per cent ammonium sulfate, and ether..	1.7	6.0*	0.7	5.8† 31.3	12.05‡	12.8	61.63		100.64
R 562C. New born calf. Residue of lean flesh composite after extraction with cold water, alcohol ether.....	1.5	5.4	0.9	4.9 33.1	13.6	13.8	1.8 60.3	58.5	100.35

TABLE I—*Concluded.*

Sample.	Nitrogen.								
	Humin.	Ammonia.	Cystine.	Histidine.	Lysine.	Arginine.	Non-amino, in filtrate.	Amino, in filtrate.	Total.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C 562C. New born calf. Coagulated protein from lean flesh composite.	2.3	6.8	1.1	5.3 30.6	13.3	10.9	 61.36		101.07
C 505. Steer, 11 mos. Coagulated protein from lean round of fat steer.	2.2	6.7	1.0	9.1 33.1	13.1	9.9	2.7 60.10		102.06
C 501. Steer, 4 yrs. Coagulated protein from lean round of fat steer.	2.1	6.9	1.2	7.6 34.8	15.5	10.2	 56.26		100.07
C 48. Steer, 5 yrs. Coagulated protein from lean round of fat steer.	2.6	6.0	0.8	5.4 30.0	12.7	11.1	0.3 60.1	59.8	97.88

* During the ammonium sulfate extraction some ammonia may have been united with the protein, making the ammonia nitrogen value too high.

† This value is too high. See discussion of the histidine nitrogen.

‡ This value is too low. See discussion of the histidine nitrogen.

The humin nitrogen is about 1 per cent lower in cold water-insoluble proteins than in the coagulable proteins. This may be due to differences in carbohydrate, proline, and tryptophane contents. Van Slyke found 1.28 per cent humin nitrogen in a hydrolysate of casein.

Ammonia Nitrogen.

The greater part of the ammonia nitrogen formed during the hydrolysis of proteins comes from the acid amides. Other sources of ammonia are the products of secondary decomposition of some of the amino-acids, such as tryptophane and cystine. In these samples of meat proteins the ammonia nitrogen is higher in the coagulated proteins than in the cold water-insoluble samples. It may be noted that Sample *R* 508B has a higher ammonia content than the other cold water-insoluble samples. The sample had been extracted with ammonium sulfate and thoroughly washed. It may be possible that some ammonia has united with the free amino groups of the protein as the acid salt, $R\text{-}NH_2(NH_4)HSO_4$.

Cystine Nitrogen.

The amounts of cystine nitrogen given in the table are lower than the true quantities present, since it is known that cystine gives rise to humin formation, ammonia formation, and that H_2S (9, 10, 11) is given off during hydrolysis. The quantities present, however, are all very close to 1 per cent and the determinations are of value in calculating the per cent of lysine. The cystine content of these proteins is slightly higher than that obtained by Van Slyke by the same method in casein, which was 0.70 per cent (corrected for solubilities of bases).

Due to the presence of silica the cystine in Samples *R* 508B and *C* 48 was not weighed. However, a very small precipitate was noticed. The values given in the table give the amount of cystine nitrogen which would not have been precipitated by the phosphotungstic acid.

Arginine Nitrogen.

The arginine nitrogen in the cold water-insoluble samples is higher than in the coagulated samples.

Lysine Nitrogen.

The values given in the table for lysine nitrogen are slightly lower in the cold water-insoluble samples. However, a correction

for nucleoprotein bases would make the values slightly higher so that it is impossible to say from these data that there is any marked difference in amount of lysine nitrogen present.

Summarizing these results we find that all of the cold water-insoluble samples differ decidedly from the coagulated samples in their hydrolytic products; that is, in the humin nitrogen formed from them and in the ammonia, the histidine, and the arginine nitrogen content. The differences among the insoluble samples as well as among the coagulated samples must be due to the different sources of the samples.

The three samples of coagulated protein from the round of Animals 505, 501, and 48, are comparable except as to age, but while the analysis shows differences these differences do not vary with the age of the animal.

Histidine Nitrogen.

The histidine nitrogen is lower in the cold water-insoluble samples than in the coagulable samples. The value given in the table for Sample *R* 508B is too high since in determining the amino nitrogen in the bases the nitrous acid was allowed to act for only 5 minutes, while it requires 30 minutes for complete action upon the ϵ-amino group of lysine. About four-fifths of the nitrogen of the ϵ-amino group of lysine is liberated by nitrous acid in 5 minutes. Allowing a correction for this, the value for histidine becomes 3.9 and that for lysine, 13.9.

Corrections for the Presence of Nucleoproteins.

The cold water-insoluble samples contain small quantities of nucleoproteins. The nucleic acid (11, 12) of the nucleoproteins contains purine and pyrimidine bases which are precipitated by phosphotungstic acid.

The purine nitrogen in Sample *R* 562C was determined according to the method of Hall (13) and found to be 0.44 per cent of the total nitrogen. Then the purine and pyrimidine nitrogen precipitated by phosphotungstic acid = $\frac{15}{10} \times 0.44$, or 0.66 per cent. The formula for calculating the per cent of histidine can be corrected for nucleic acid bases and will be: histidine nitrogen = $\frac{3}{2}$(D − $\frac{3}{2}$ purine nitrogen − $\frac{3}{4}$ arginine nitrogen); and for lysine

nitrogen: lysine nitrogen = total nitrogen of bases $-\frac{3}{2}$ purine nitrogen $-$ arginine nitrogen $-$ histidine nitrogen $-$ cystine nitrogen.

Using these formulas, the value of the histidine nitrogen in Sample *R* 562C becomes 3.93 and for lysine nitrogen, 13.89. The correction lowers the amount of histidine by 1.0 per cent and raises the amount of lysine by 0.33 per cent. Several assumptions have been made in these calculations, but it is certain that nucleic acid in the proteins which are analyzed according to Van Slyke causes the determination of histidine to be too high and that of lysine to be too low. This fact strengthens the conclusion that the cold water-insoluble samples have less histidine nitrogen than the coagulated samples.

The non-amino nitrogen of the filtrate from the bases represents one-half of the tryptophane and all of the proline and oxyproline nitrogen. The differences shown in the table are not significant because slight errors in the determination will change the results appreciably. In some cases no non-amino nitrogen is given in the table because the amino nitrogen determination was slightly higher than the total nitrogen determination. This does not mean that no non-amino nitrogen is present, but that the accuracy of the determinations is such that it could not be determined. However, it is certain that in the samples analyzed here the amount of non-amino nitrogen in the filtrate from the bases is not more than about 3 per cent. It may also be remarked that this form of nitrogen—tryptophane, proline, and oxyproline—makes up the largest part of the humin nitrogen (14).

DISCUSSION.

The proteins of muscle are generally divided into two groups, those of the muscle plasma and those of the muscle stroma. The greater part of the proteins of the muscle are insoluble in ammonium chloride solution. These are the proteins of the stroma. Nucleoproteins and phospholipins are also present. The latter are apparently in union with the proteins. The protein is probably an albuminoid or a metaprotein. The heat-coagulable proteins used in this work are of course all plasma proteins, while the cold water-insoluble residues contain all of the original stroma proteins and some of the plasma proteins.

Mathews (12) says: "It seems probable from the general phenomena of the squeezing out from gels on contraction of the latter by the process called syneresis, of a solution containing the same matter as the gel, but in different concentration, that the plasma may, in reality, contain the same proteins as the stroma, but in more dilute form."

The differences in constitution of the cold water-insoluble and the coagulable samples found in this work are evidence against this probability. The amount of nucleoproteins present does not have an appreciable effect upon the results of the analysis. We have here differences between mixtures of stroma proteins with plasma proteins, and plasma proteins.

Samples R 562C and R 549.—The analysis of these samples shows differences that are greater than the experimental errors. Sample *R* 562C is the cold water residue of the flesh composite of a new born calf, while *R* 549 is a composite of the lean flesh of the ribs of a 5 year old steer. The latter contained some fatty tissue. It has been found in the analysis of many beef animals in this laboratory that the analysis of the lean flesh of the ribs corresponds very closely to the analysis of the lean and fat composite of the carcass. It may be said, then, pending analysis of more samples, that it is probable that during growth a change takes place in the composition of the cold water-insoluble protein of the flesh of beef animals; the products of hydrolysis contain less ammonia nitrogen, less histidine nitrogen, and more arginine nitrogen.

Analysis of a Flesh Composite of a New Born Calf.

The analysis given in Table II of a flesh composite of a new born calf was made in order to show the relation between the proteins studied in the discussion above to the fresh flesh. Several gaps in the methods for tissue analyses are evident. The value given for the alcohol-ether-soluble nitrogen of the cold water-insoluble fraction of the flesh may differ slightly from the true value, since a small part of the sample was removed from it, while it was standing in alcohol, without previous mixing. Inspection of the table will show that no separations within the alcohol-ether-soluble nitrogen of the insoluble residue were made. The monoamino-acid nitrogen in the soluble and coagulated proteins has not been

TABLE II.

Analysis of Flesh Composite of a New Born Calf, No. 562C.

Moisture. 76.81 per cent fresh substance.			
Ether extract. 4.63 per cent fresh substance.			
Nitrogen. 2.598 per cent fresh substance.	Cold water-insoluble; 81.02 per cent of total N.	Ether-alcohol-soluble; 9.38 per cent of total cold water-insoluble N.	Total N of protein: *per cent* Humin.... 1.5 Ammonia. 5.4 Cystine... 0.9 Histidine. 4.9 Lysine....13.6 33.1 Arginine..13.8 Monoamino-acid.60.3 Total..100.0 Purine N. 0.44
		Protein nitrogen; 90.62 per cent of total cold water-insoluble N.	
	Cold water-soluble, heat-coagulable; 6.85 per cent of total N.	Total N of protein: *per cent* Humin ... 2.3 Ammonia. 6.8 Cystine. 1.1 Histidine. 5.3 30.6 Lysine....13.3 Arginine..10.9 Monoamino-acid.62.1 Total..101.7	
	Cold water-soluble, non-heat-coagulable; 12.13 per cent of total N.	Nitrogen precipitated by tannic acid, 2.04 per cent of total N.	
		Nitrogen as creatine and creatinine, 4.23 per cent of total N.	

TABLE II—*Concluded.*

Ash. 0.983 per cent fresh substance.	Cold water-soluble; 0.71 per cent of fresh substance.	Phosphorus	Organic, 0.063 per cent fresh substance. Inorganic, 0.0617 per cent fresh substance.
	Cold water-insoluble; 0.273 per cent of fresh substance.	Phosphorus, 0.047 per cent fresh substance.	

separated. However, approximate values for tyrosine and tryptophane are given on pages 357 and 360 respectively. No separations of the proteose and peptone nitrogen which is precipitated by tannic acid were made. This portion amounts to only 2 per cent of the total nitrogen of the flesh. The only constituent of peptide and extractive nitrogen which was determined was creatine and creatinine nitrogen which amounts to 42 per cent of the total peptide and extractive nitrogen.

The methods used in these analyses are those described or referred to in this and the subsequent paper.

CONCLUSIONS.

1. Cold water-insoluble proteins of beef flesh yield on hydrolysis less humin, ammonia, histidine, and more arginine nitrogen than coagulated beef proteins. A possible significance of these results is discussed.

2. From the analyses of cold water-insoluble samples from the flesh of a new born calf and of a 5 year old steer it seems probable that during growth the insoluble proteins change in composition. The products of hydrolysis contain less ammonia, less histidine, and more arginine nitrogen.

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DETERMINATION OF VARIOUS FORMS OF NITROGEN IN BOVINE FLESH, INCLUDING THE PRODUCTS OF HYDROLYSIS OF SOME OF THE PROTEINS.

SECOND PAPER.

THE BROMINATION OF THE HYDROLYSATES OF SOME BEEF FLESH PROTEINS.

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The tyrosine in some of the samples described in the foregoing paper was determined according to the method of Plimmer and Eaves (1). The method depends upon the fact that tyrosine absorbs two atoms of bromine. The bromine is prepared according to the equation: $NaBrO_3 + 5NaBr + 6\,HCl = 3Br_2 + 6NaCl + 3H_2O$. An excess of 0.2 N $NaBrO_3$ solution + NaBr solution is added and after 15 minutes absorption the solution is treated with NaI and the iodine which corresponds to the excess bromine is titrated against 0.1 N $Na_2S_2O_3$, using starch as indicator. Protein and some of its constituents besides tyrosine, histidine, cystine, and tryptophane absorb bromine. Tyrosine is separated from proteins, histidine and cystine by precipitation with phosphotungstic acid, and separation from tryptophane is effected by a trypsin hydrolysis since the tryptophane of a protein does not appear in the early stages of trypsin digestion. Plimmer and Eaves have settled upon 6 hours of digestion with tyrpsin as the length of time required by trypsin to liberate the tyrosine but not the tryptophane of a protein. They found a close agreement between the figures for tyrosine content obtained by this method from a large number of proteins and the figures obtained by isolation and weighing.

The technique used in the analyses presented here is as follows: 1.25 gm. of the air-dry protein in a 250 cc. beaker were treated with 10 cc. of 23.8 per cent NaOH solution and 50 cc. of water and boiled for a short time. All but a very small amount of protein went into solution. 4.7 cc. of 33.7 per cent solution of HCl were added slowly with stirring. The volume of the solution was brought up to 100 cc., giving it a concentration of 0.2 per cent NaOH. The solution was warmed to about 37° and 0.1 gm. of trypsin was added. 2 cc. of chloroform were added to prevent bacterial action, and the mixture was allowed to digest exactly 6 hours at 37° in an incubator. The solution was then quickly transferred to a 110 cc. sugar flask, cooled to room temperature, and made up to volume. Two 50 cc. aliquots were treated in a 250 cc. beaker with exactly 100 cc. of a 10 per cent phosphotungstic acid in 5 per cent sulfuric acid solution. After stirring, the precipitate consisting of proteins and basic amino-acids was filtered through dry folded filters. 100 cc. of the filtrate were run into glass-stoppered flasks and brominated as follows:[1] 10 cc. of a 0.2 N $NaBrO_3$ solution were run into the flask from a burette and then 10 cc. of a 2 per cent NaBr solution were added. The stopper was put into the flask and the flask was gently rotated and allowed to stand for 15 minutes in diffused light. It was then cooled under the water faucet and a few drops of a 4 per cent NaI solution were placed in the depression above the stopper of the flask. When gently opened the slight vacuum in the flask will draw in the NaI solution which will absorb any bromine escaping. 10 cc. of the NaI solution are added and the stopper is replaced and the flask gently rotated. Then the iodine solution on the stopper and neck of the flask is rinsed with water into the flask and the contents of the flask are titrated against 0.1 N thiosulfate solution, using starch as indicator. Sometimes the presence of the phosphotungstic acid makes it difficult to recognize the end-point. This is especially so if the original protein solution is dark. In that case the end-point is a change from a distinct blue to a violet. It is, however, readily found after some experience with an error no greater than three drops.

A blank determination on 0.1 gm. of trypsin was also made. The thiosulfate solution was titrated against 0.1 N potassium dichromate. The blank titration minus the titration of the samples gives the amount of bromine absorbed by the sample in terms of 0.1 N thiosulfate. Table I gives the results of these determinations upon the six samples analyzed.

The results of the tyrosine determinations show variations from 0.82 to 0.92 per cent of tyrosine nitrogen in terms of total nitrogen of the proteins, or 1.69 to 1.91 per cent tyrosine in the proteins. An error of one drop in titration is equal to 0.018 per

[1] Plimmer and Eaves recommend the use of NaI and NaBr instead of KI and KBr because phosphotungstic acid is precipitated when K salts are present, making titration difficult.

cent tyrosine nitrogen. These differences are probably within experimental errors, and we may say that the tyrosine nitrogen in all these proteins in terms of total nitrogen is close to 0.9 per cent, or that the tyrosine content of these proteins is close to 1.9 per cent. This latter figure is somewhat lower than the one given for ox muscle protein obtained by isolation and weighing of the tyrosine by Osborne and Jones (2) who obtained 2.2 per cent tyrosine. Plimmer and Eaves found differences as great as that by the two methods.

The figures given were calculated upon the assumption that tyrosine absorbs two atoms of bromine (four atoms used) under the conditions of analysis. Plimmer and Eaves obtained very

TABLE I.

Sample.	Thiosulfate used.	Tyrosine N as total N.	Tyrosine in protein. Wt. of protein = 6.25 × wt. of N of sample.
	cc.	*per cent*	*per cent*
**R* 549	2.25	0.955	1.98
**R* 508 B	2.05	0.904	1.87
**R* 562 C	1.91	0.82	1.72
†*C* 562 C	2.02	0.924	1.91
†*C* 505	1.82	0.818	1.69
†*C* 501	2.07	0.907	1.89

* Cold water-insoluble residue.

† Coagulated sample.

nearly theoretical results under these conditions. However, when a great excess of bromine is present tyrosine absorbs more bromine. Siegfried and Reppin (3), who also give valuable hints on the technique of bromine titrations, found that about four and one-half molecules of bromine were used by tyrosine with varying amounts of bromine excess and under conditions not greatly different from those of this work.

The coagulated Sample 562C absorbed a small amount of bromine when treated as described but without the addition of trypsin. Coagulated Sample 505 after 24 hours digestion showed no increase in bromine absorption. Sample *R* 549 after 72 hours digestion showed a marked increase in bromine absorption. The results given here are not to be considered as absolutely reliable

because the method of analysis depends upon several assumptions. It is assumed that all of the tyrosine is split off during 6 hours of tryptic digestion and that none of the tryptophane has been liberated during that time. The verity of these assumptions has so far only been proved in the case of nine vegetable proteins and of casein by Plimmer and Eaves. In these samples they obtained more or less close agreement between the bromination and the isolation method of determining tyrosine. However, if there were any great differences in the tyrosine content of these proteins this method of analysis would show them.[2]

Bromination of Hydrolytic Products of Beef Flesh Proteins.

Siegfried and Reppin (3) found that unhydrolyzed proteins absorb more bromine than their hydrolytic cleavage products. They found also that the following acids did not absorb bromine except after long standing: glycocoll, sarcosine, betaine, alanine, valine, leucine, aspartic acid, glutaminic acid, lysine, arginine, phenylalanine. By treating tyrosine with an excess of bromine and titrating back with starch and iodine, one molecule of tyrosine absorbed four and a half atoms of bromine, one of tryptophane about eight atoms, and one of histidine somewhat over two atoms. Cystine consumes bromine and the destruction of it and the tryptophane in acid hydrolysis of the proteins may account for the fact that unhydrolyzed proteins absorb more bromine than their hydrolysates. However, Siegfried and Reppin found that edestin before trypsin hydrolysis absorbed more bromine than after. The doubtful explanation they offer is that the increased bromine consumption due to liberation of tyrosine is more than balanced by the destruction of bromine-consuming linkings during hydrolysis.

Brown and Millar (4) and Millar (5) found an increase in capacity for bromine absorption during the progress of protein hydrolysis, which they attributed to the liberation of tyrosine which is not supposed to absorb bromine in a combined form. However, their results were obtained under different conditions. No excess of bromine was added. Bromate solution was added to the solution of the sample until the latter gave a color reaction with starch-iodine solution.

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[2] Since the writer was unable to find any data in the literature on the bromination of cystine a determination of the bromine consumption of cystine was made under the same conditions which governed the other bromination experiments reported in this paper. An impure specimen of amino-acid (88.5 per cent) absorbed the equivalent of 10 atoms of bromine. The reaction was not further investigated.

Method for Determining Histidine.

The following experiments were made to determine whether or not bromine absorption determinations would be of value in conjunction with the Van Slyke analysis. Two 5 cc. aliquots (= 0.0323 gm. of protein nitrogen) of the solution of the bases of the coagulable protein sample, No. 505, were treated with 10 cc. of bromate solution (= 18.71 cc. thiosulfate) and 50 cc. of water, 10 cc. of bromide solution, and 5 cc. of HCl. Bromination was allowed to proceed for 15 minutes. It took 17.0 cc. of thiosulfate (= 0.008465 gm. of bromine) to titrate the excess of bromine (see p. 356 for technique). The bromine absorbed was 0.01477 gm. Deducting 0.00259 due to cystine (cystine nitrogen = 0.0009 found by determining sulfur in another aliquot), assuming that cystine consumes ten atoms of bromine per molecule, we have a consumption of 0.0119 gm. Br_2 by the histidine in solution. One molecule of histidine absorbs two atoms of bromine according to Siegfried and Reppin. Calculating the per cent of histidine nitrogen from the above data we get 9.68 per cent histidine nitrogen. To this must be added 1.17 per cent as a correction for solubility of histidine in presence of phosphotungstic acid as determined by Van Slyke, making it 10.85 per cent histidine nitrogen. This is somewhat higher than was obtained by the Van Slyke method, 9.08 per cent. By the same method Sample *R* 562C had an average of 4.73 per cent histidine nitrogen; by the Van Slyke method, 4.97 per cent. The method also gave a value of 2.15 per cent histidine nitrogen for Sample *R* 549, while the Van Slyke method gave 1.90 per cent.

The accuracy of this method may be considerably increased by using more dilute solutions for the titrations so as to measure larger volumes. A factor for bromine absorption for histidine may be of value since histidine absorbs somewhat more than two atoms of bromine per molecule.

Attention may be called to the fact that this method requires only two determinations of which the cystine nitrogen is very accurate, while the Van Slyke method requires three determinations. Amyl alcohol may interfere by absorbing bromine, but in the Van Slyke analysis the solution of the bases after removal of the phosphotungstic acid is evaporated under a vacuum and

boiled off. It is not known whether the purine and pyrimidine which are precipitated by phosphotungstic acid will absorb bromine under the conditions employed in these experiments. If further proof of the value of this method for the determination of histidine is obtained a Van Slyke apparatus will no longer be essential for a determination of the hexone bases.

Bromination of the Filtrate from the Bases.

Table II gives the results of brominating 50 cc. of the filtrates from the bases in the Van Slyke determinations. The end-point due to the color of the phosphotungstic acid is difficult to recognize until one has had some experience, when it can be recognized within a drop or two of the thiosulfate solution.

TABLE II.

Bromination of Filtrate from Bases.

Sample.	*R* 562C	*R* 549	*C* 505	*C* 501
Br absorbed as thiosulfate, *cc.*	8.81	11.06	10.76	11.46
Br absorbed after subtracting 0.01483 gm. for cystine and 0.00289 gm. for histidine in solution, *gm.*	0.05686	0.07590	0.07129	0.07929
Br absorbed by tyrosine in same weight of sample, *gm.*	0.01650	0.01950	0.01215	0.01845
Br absorbed by tryptophane, *gm.*	0.04056	0.05640	0.05914	0.06084
Nitrogen in sample, *gm.*	0.07831	0.08951	0.06460	0.09548
Tryptophane nitrogen calculated for a bromine absorption of 6 atoms per molecule, *per cent.*	3.02	3.68	5.35	3.72
Tryptophane, *per cent.*	3.53	4.30	6.24	3.45

This bromine absorption may be due to the tyrosine and the tryptophane which have escaped destruction during the acid hydrolysis and the histidine and cystine which have escaped precipitation by the phosphotungstic acid.

The following experiments were made to determine whether or not the correction for the solubility of the cystine can be applied to brominations of the filtrate.

Two aliquots of a cystine solution which would absorb bromine equivalent to 9.25 cc. of thiosulfate were treated in a volume of 50 cc. with 4 gm. of phosphotungstic acid and 5 cc. of HCl and heated on a water bath. The solution was allowed to cool and after 36 hours standing the precipitate was filtered off with suction and washed with phosphotungstic acid solution. The filtrate was brominated and consumed an amount of bromine equivalent to 2.9 cc. of thiosulfate. The phosphotungstic acid was removed from the precipitate by extracting it according to Van Slyke with amyl alcohol and ether. The solution of the precipitate after subtraction of a blank (2.26 cc.) due to ether and amyl alcohol in the solution absorbed bromine equivalent to 6.7 cc. of thiosulfate. The precipitate and the filtrate together consumed bromine equivalent to 9.6 cc. of thiosulfate. The slight excess (0.35 cc.) may be due to inaccuracy of the blanks determined for the bromine consumption of the ether and amyl alcohol in the solution containing the precipitated cystine. Phosphotungstic acid does not consume bromine, as solutions of bromine titrate the same amount of thiosulfate in the presence of phosphotungstic acid as without it. If a solution of phosphotungstic acid is colored due to formation of oxides between WO_2 and WO_3, bromine will destroy the color, but upon titration with thiosulfate it appears again, apparently not affecting the final bromine consumption. The amount of cystine used in the above experiment contained 0.002502 gm. of nitrogen. According to Van Slyke, $\frac{0.0026}{4}$ or 0.00065 gm. of cystine nitrogen should be in the filtrate from the phosphotungstic acid precipitate. This would be equivalent to a bromine consumption of 2.4 cc. in terms of the thiosulfate. The bromine actually consumed was 2.9 cc., a fair agreement with the theoretical.

If now the correction for unprecipitated cystine and histidine is made in the bromine absorption determinations upon the filtrates from the bases, we have the weight of bromine absorbed by the tyrosine and the tryptophane. The amount of bromine absorbed by the tyrosine has been determined by the method of Plimmer and Eaves on these proteins. Subtracting the bromine absorption due to tyrosine from the corrected value for tyrosine plus tryptophane, we get the bromine absorption due to tryptophane. The bromine absorption by tryptophane varies with the time and the excess of bromine added from about six atoms of bromine per molecule according to Plimmer and Eaves (1), Homer (6), and Siegfried and Reppin (3). The table shows the values for tryptophane nitrogen calculated on the assumption that one molecule of tryptophane absorbs six atoms of bromine. These values are only offered as indicating the approximate amount of tryptophane in the proteins, as some tryptophane is destroyed during hydrol-

ysis, and because the method of Plimmer and Eaves is not as reliable as is desirable. The values for Sample *R* 562C were calculated on the assumption that the tyrosine content as determined by the Plimmer and Eaves method was too low and that 2.2 per cent as found in ox muscle by Osborne and Jones (2) by isolation and weighing was the true tyrosine content. It was found that the 0.5 per cent difference in tyrosine content reduced the value for tryptophane nitrogen by about 0.3 per cent and the tryptophane by about 0.4 per cent.

CONCLUSIONS.

1. The tyrosine nitrogen was determined according to the method of Plimmer and Eaves in a number of cold water-insoluble and a number of coagulated beef proteins. No significant differences were found. The reliability of these determinations is discussed.

2. The amount of bromine which cystine will absorb when treated with an excess of nascent bromine for 15 minutes was found to be about ten atoms per molecule. Cysteic acid is probably the end-product of the oxidation of cystine by bromine under these conditions.

3. A new quantitative method of determining histidine in proteins by brominating the solution of the bases has been developed. Determinations of histidine on three different proteins by this method agree well with the results obtained by the Van Slyke method.

4. The filtrate from the bases was brominated. From these data approximate values for the tryptophane content were calculated.

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THE BASIS OF MEASUREMENT OF ANTAGONISM.

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(Received for publication, March 6, 1918.)

In previous papers[1] the writer has described a method of measuring antagonism. The essential feature of this method is the use of the additive effect. If this is accurately known we can measure even slight antagonism. This will be made clear by a reference to Fig. 1. In this figure the ordinates represent growth while the abscissæ represent the molecular concentrations of two salts, A and B. In this case the two salts are equally toxic (as shown by the fact that the ordinates at the extreme left and extreme right are equal). If the toxic effects of both salts are similar and if neither influences the action of the other, we may replace a molecule of A by a molecule of B without changing the effect on the plant, and it will make no difference whether we have A 0.90 M + B 0.10 M or A 0.50 M + B 0.50 M. In any mixture the effect will be the same as in any other or in either of the pure salts. This we call the additive effect. It is defined as the effect which the salts would bring about if they acted in a similar manner and independently.

As was pointed out in former papers, any growth in excess of the additive effect may be regarded as antagonism while anything less than the additive effect may be regarded as the opposite of antagonism.

The antagonism at any point, as at the ordinate I, may be measured in two different ways. In the first method we regard the additive effect as the distance HI. The antagonism would then be the excess of growth HF, over the additive effect.

It is quite possible that if all the solutions were diluted with an equal volume of water the antagonism might remain unchanged.

[1] Osterhout, W. J V., *Bot. Gaz.*, 1914, lviii, 178, 272; 1916, lx, 228.

It is probable that the growth would be greater in all of the solutions and that the distance HI would be increased. It is therefore evident that in this case antagonism should be expressed as per cent of the additive effect or as $100 \times FH \div HI$. The opposite of antagonism, as shown by the lowest curve, would be expressed as $100 \times EH \div HI$.

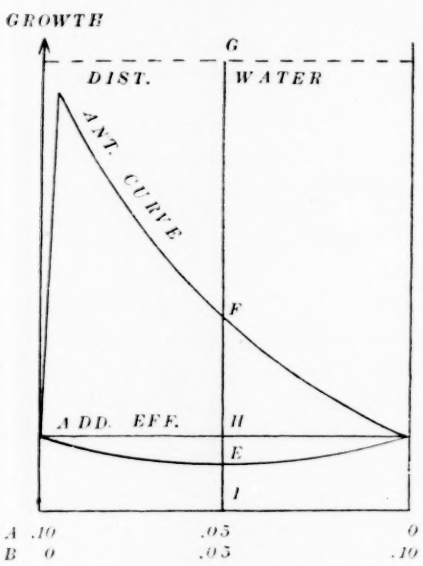

FIG. 1.

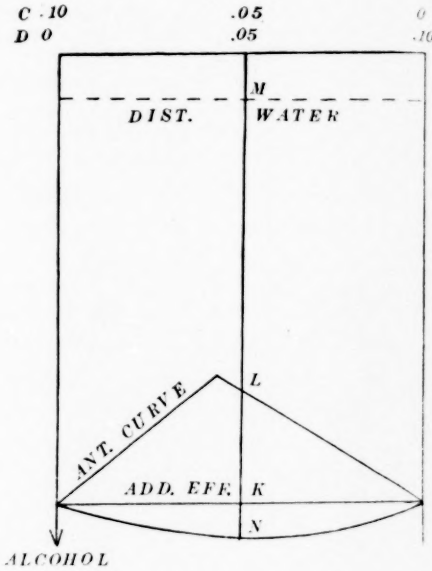

FIG. 2.

FIG. 1. Curve showing antagonism between two salts, A and B. The additive effect is GH. Antagonism at the ordinate I may be expressed as $100 \times FH \div GH$; the opposite of antagonism as $100 \times EH \div GH$. The dotted line represents growth in distilled water. The abscissæ represent the molecular concentrations of the salts.

FIG. 2. Curve showing antagonism between two salts, C and D, in their effect on the precipitation of gelatin by alcohol. The ordinates, measured from above downwards, represent the amount of alcohol necessary to precipitate gelatin containing salts whose molecular concentration is represented by the abscissæ. The dotted line represents the amount of alcohol necessary to precipitate gelatin to which no salts have been added (but containing the same amount of distilled water as the rest of the gelatin). The additive effect is KM. Antagonism may be expressed as $100 \times LK \div KM$, the opposite of antagonism as $100 \times KN \div KM$.

In the second method we look at the matter from the following standpoint. A certain amount of growth occurs in distilled water; when salts are added to distilled water the growth is lessened. The lessening of growth due to the action of the salts where no antagonism (or its opposite) occurs is regarded as the additive effect. When the salts are antagonistic growth is less hindered. The additive effect is then GH; the antagonism is FH and may be expressed as $100 \times FH \div GH$. The opposite of antagonism is $100 \times EH \div GH$.

The second method of measurement has certain advantages. This is evident, for example, when we attempt to compare antagonistic effects produced on protoplasm (Fig. 1) with those produced on gelatin (Fig. 2). In experiments on gelatin Fenn[2] found antagonism between various salts by testing their effect on the precipitation of gelatin by alcohol. In Fig. 2 the ordinates (measured from above downward) represent the amount of alcohol required to precipitate a given amount of gelatin; the abscissæ have the same significance as in Fig. 1. It is evident that we cannot use the first method of measurement here, but that the second can be applied without difficulty. Using this method, antagonism at the point K is $100 \times LK \div KM$.

In this instance the additive effect is easily determined, but in some cases there may be difficulties. The additive effect can always be determined when it is possible to express the effect of one salt in terms of the other, as is the case in Fig. 3 (the effect in distilled water is here the base line). Here it will be seen[3] that the amount of alcohol required in the presence of P is the same as that required in twice the same concentration of R, so that we may write, amount in (0.50 P + 0.50 R) = amount in (0.50 P + 0.25 P) = amount in 0.75 P = 20, or amount in (0.50 P + 0.50 R) = amount in (1.00 R + 0.50 R) = amount in 1.50 R = 20.

Whenever the effect of one salt is everywhere a constant multiple of that of the other the additive effect can be accurately determined. Even when such a relation is only approximated, the approximate additive effect forms a useful basis of measurement.

[2] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiv, 141.

[3] In this case the effect in distilled water is taken as the base line. In place of molecular concentrations we may use for abscissæ the number of cc. of the solution which are taken in making the mixture.

When the approximation is not close, special methods of calculation may be employed, as explained in a previous article.[4]

When the additive effect cannot be determined with sufficient accuracy to be of value, we may find it impossible to say whether antagonism is present unless the following criterion can be applied. It is evident that the additive effect as measured by the second method must always be greater than that of the most strongly acting salt. We are therefore safe in saying that antagonism exists if the effect of any combination of substances is less than that pro-

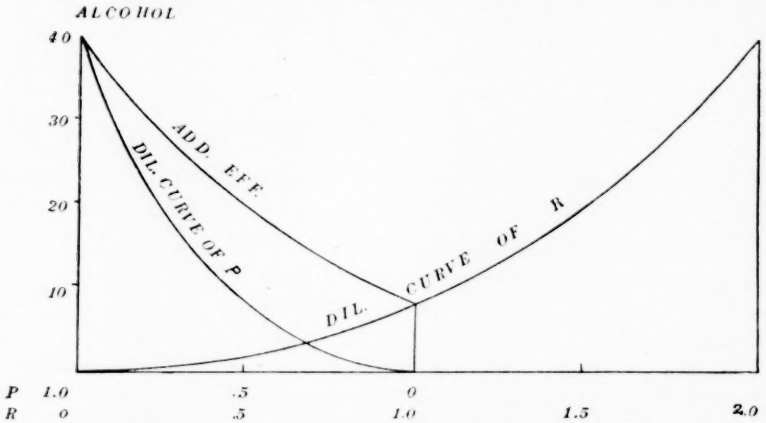

FIG. 3. Curves showing the effects of dilution and the additive effect of two salts, P and R. The ordinates represent the amount of alcohol required to precipitate gelatin to which the salts have been added, the abscissæ represent molecular concentrations. In the dilution curve of P the abscissæ represent molecular concentrations of P (R being absent); similarly in the curve for R (P being absent).

duced by the most strongly acting substance in the absence of the others (and at the same concentration at which it exists in the combination). If the effect is greater than this it may be impossible to decide (the additive effect being unknown) whether we have antagonism, additive effect, or the opposite of antagonism. In cases where the effect of one salt is very small it may be neglected altogether. If only two salts are present the addi-

[4] Osterhout, *Bot. Gaz.*, 1916, lx, 228.

tive effect will then be practically the same as that of the most strongly acting salt.

It may be of interest to consider briefly cases where the concentration of one salt is kept constant while that of the other is varied. Such a case is represented in Fig. 4. Here it is evident that antagonism exists as long as the curve of growth is above the

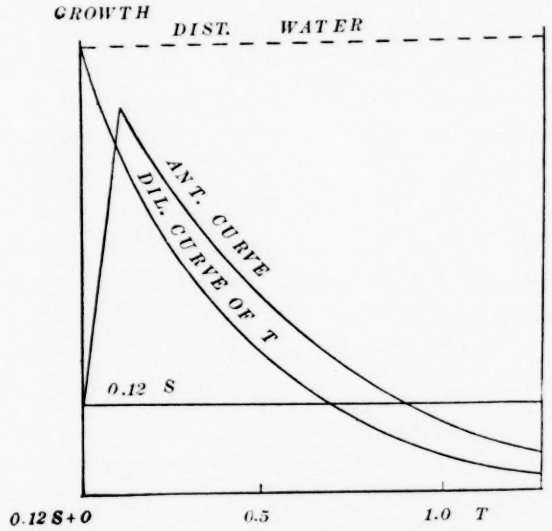

FIG. 4. Curve showing antagonism between two salts S and T. The concentration of S is constant (0.12 M) while that of T varies as shown by the abscissæ (which represent molecular concentrations of T). The effect of S 0.12 M is shown by the horizontal unbroken line. The effect of various concentrations of T alone is shown by the dilution curve of T; for this curve the abscissæ represent molecular concentrations (S being absent). Growth in distilled water is shown by the dotted line.

horizontal line which indicates the growth in 0.12 S. But where it drops below, as it does to the right, we must know the effect of T in order to decide whether antagonism exists. This effect is shown by the dilution curve of T in the figure. In order to calculate the additive effect the dilution curve of S must also be known.

It is hardly necessary to add that all the methods discussed above are applicable when more than two salts enter into the mixture.

In some cases distilled water may not be satisfactory as a standard of comparison. In the case of marine organisms the natural medium to use is sea water. In this case, just as where distilled water is used as a basis, the organism itself contains a variety of salts, and the experimenter merely increases the amount of one of them so that it predominates. In the case of distilled water this can be done only by adding the salt, while in the case of a constituent of sea water it can be done by adding this salt or by diminishing the other constituents or by doing both simultaneously.[5] In such cases we obtain curves similar to those shown in Fig. 1, except that the effect of sea water is substituted for that of distilled water as the standard of comparison.

It would not be safe to extend this principle to the substitution of nutrient solutions for distilled water, for here the antagonistic effects might be confounded with nutrient effects, at least where growth is used as a criterion. It may be that other criteria can be found which are not affected by nutrition to so great an extent as growth is, and it is possible that permeability might be used in this way. But it should be noted that when the experimenter adds toxic salts to a nutrient solution he must make due allowance for the antagonistic effect of the salts already present.

SUMMARY.

In order to measure antagonism it is necessary to know the additive effect (the effect which would be found if no antagonism existed).

When the additive effect is unknown it may be impossible to decide whether antagonism exists unless the observed effect is less than that of the most strongly acting substance (measured from the distilled water curve which is taken as a base line). If the observed effect is greater than this it may be due to antagonism, to additive effect, or to the opposite of antagonism.

Methods of determining the additive effect and of measuring antagonism are discussed.

[5] The same principle applies when the salt used is not a constituent of sea water, the organism being placed in a solution of this salt in distilled water.

DETERMINATION OF HEMOGLOBIN DURING INFANCY BY THE PALMER AND VAN SLYKE METHODS.

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Many authors have pointed out the high hemoglobin content of blood of new born infants. Leichtenstern (1) was one of the first to call attention not only to the high value of hemoglobin at birth but to the rapid decrease in the early days of life. Schiff (2) using the Fleischl apparatus found 104.6 per cent hemoglobin at birth with a drop of 14 per cent in the first 14 days. Gundobin (3) with the same instrument obtained 90 to 115 per cent hemoglobin in the new-born. Takasu (4) using the Gower hemoglobinometer gives 103 to 160 per cent as the figures for normal infants during the first few days of life. Williamson (5) has shown the variations according to age by spectrophotometric analysis amplifying and confirming the earlier work of Leichtenstern. There is a physiological decrease in hemoglobin during the first 2 years of life which is very rapid during the first 5 or 6 months. The hemoglobin content does not again increase until after the 2nd year. The cause for this physiologically low hemoglobin during infancy is not clear and may be closely related to the anemia of infancy seen especially in infants born prematurely and in twins.

Because of the wide range in the results obtained by the above authors, it appeared desirable to ascertain the hemoglobin in the blood of normal infants by the method of Palmer (6), which is capable of 1 per cent accuracy. It is also sufficiently simple to be available as a practical clinical method and has the advantage of using a standard solution, any deterioration of which can be readily detected by checking readings with the results obtained by the gasometric method described by Van Slyke (7).

Selection of Cases.

The blood of a series of 103 children less than 2 years of age was studied by the Palmer method. The Van Slyke method was employed in only a few cases, being used to check the Palmer

method. The results by the two methods corresponded very closely. Fine, healthy children were chosen, showing normal weight with steady gain, good development, both physical and mental, and absence of any disease, acute or chronic, of any consequence at the time the specimen was taken or previously. About 90 per cent were breast-fed. Except in new born babies nearly all of the examinations were made during the late summer or autumn at a season when the children were out in the fresh air a great deal.

Collection of Blood Samples.

The blood was taken from the toe, usually both great toes being used to procure an adequate amount of blood quickly. To aid drop formation in the blood of new born infants a thin film of sterile vaseline was applied after the toe had been cleansed with alcohol. The samples of blood were collected and measured in a special pipette made of heavy capillary glass tubing of 1 mm. bore. The pipette was bent at right angles about 8 mm. from the tip so that the blood could be drawn up by capillary attraction. While the pipette was being filled it was held in a horizontal position so that capillary attraction had to overcome only the weight of the short vertical column at the tip. By lowering or raising the stem from the horizontal position the inflow of blood could be accelerated or retarded. The pipettes were calibrated gravimetrically with water to contain 0.05 and 0.10 cc., each pipette being calibrated to be used either wet or dry. Care was taken to drain always in the same manner, when, as in most cases, the pipettes were used wet.

Standard Hemoglobin Solution.

Dog or human blood was used for making the standard hemoglobin solution. A 1 per cent solution was checked every week or two by comparison with the gasometric method and was found to begin to deteriorate at room temperature in 2 to 4 weeks. Marked deterioration became readily evident by the change of color from red to yellow when the carbon monoxide hemoglobin was viewed in the colorimeter, but the initial stages of change could be detected only by comparison with the gasometric method. Differences of 5 per cent between results obtained by the colorimetric and gaso-

metric methods could be noted in a deteriorating standard before the change in color was visible.

The blood used in the gasometric method was oxygenated in a large test-tube by bubbling air through it.

Age	1 day.	2 to 3 days.	4 to 8 days.	9 to 13 days.	2 to 8 weeks.	3 to 5 months.	6 to 11 months.	11 to 23 months.
No. of cases	12	14	14	8	16	10	17	12
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Hemoglobin in individual cases.	141	122	122	114*	81	75*	73	60
	143	128	122	117	84	76*	75*	75*
	147*	137	122	128	85*	80*	77	80
	156*	138*	123	131*	88*	86*	77	84*
	157*	140	123	132*	91*	88*	79	86
	166	143*	128	144*	94	88*	82	86*
	166	147*	128	156*	97	91	83	87*
	172	147*	132	174*	100	92	87*	87*
	172	148	133*		101*	100	90*	91
	177	149*	148*		103	103	90*	91
	178*	156	150		103		90	93
	192*	156	152		104*		91	94*
		165*	152		109*		91	
		170*	157		115*		93*	
					137		93*	
					143		97	
							111	
Average	164	146	135	137	102	88	87	85

* Average of duplicates.

RESULTS.

The values found are arranged in the accompanying table according to the age grouping of Williamson. The results are expressed as percentages of hemoglobin, taking as 100 per cent Haldane's average for normal adults; *viz.*, the hemoglobin content corresponding to an oxygen-binding capacity of 18.5 cc. of oxygen per 100 cc. of blood. The early rapid decrease and later more gradual decrease occur as found by previous authors. The variations in children of the same age are noticeable in the early days of life. These data from normal children, taken with those

of previous authors, emphasize the necessity of interpreting hemoglobin determination in infants with the values normal for the age of the subject, rather than those normal for an adult, as standard.

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MONTHLY METABOLISM OF NITROGEN, PHOSPHORUS, AND CALCIUM IN HEALTHY WOMEN.*

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(Received for publication, March 8, 1918.)

For several years there has been growing appreciation of the importance of adequate intake of phosphorus and calcium, and general recognition of the fact that a freely chosen food supply, even if of ample fuel value and protein content, does not always furnish enough of these inorganic elements. There have, however, been relatively few attempts to establish the phosphorus and calcium requirements of human nutrition quantitatively, and such experiments as have been made with this in view have been almost entirely upon male subjects.

Experiments upon women were therefore plainly needed and since the effect of the monthly cycle upon phosphorus and calcium metabolism had not been determined, it seemed especially desirable that the experiments should be so planned as to throw light upon this question in addition to furnishing data upon the quantities of calcium and phosphorus required in normal human nutrition. For this purpose two healthy women (graduate students) undertook to live upon prearranged diets absolutely uniform from day to day, with quantitative determination of intake and output of nitrogen, phosphorus, and calcium in ten successive periods of 3 days each. The feces belonging to successive periods were marked by taking analyzed lampblack or carmine with the first meal of each period.

* The experiments described in this paper were made possible by the cooperation of the Department of Social Welfare, New York Association for Improving the Condition of the Poor, to which we are indebted for a grant from the research fund established by Mrs. Elizabeth Milbank Anderson.

Since each diet was to be entirely without variation from day to day for a month, the foods were selected according to the preference of each subject in as far as this was consistent with the necessary degree of simplicity. It was also a part of the general plan to secure uniformity of intake by preparing as much as possible of the food in advance, and to arrange a ration which was expected to satisfy the requirements for energy and protein while sufficiently low in phosphorus and calcium to test the ability of the body to establish equilibrium of either of these elements. The subjects drank only distilled water. Throughout the time covered by the experiments the subjects were engaged in laboratory work—usually about 10 hours per day.

Subject K weighing 52 kilos took a simple form of "ordinary mixed diet" consisting of bread, butter, beef, orange juice, shredded wheat, and cream, which furnished daily about 2,137 calories, 62 gm. of protein, 0.70 gm. of phosphorus, and 0.30 gm. of calcium. Subject L weighing 57 kilos took a meat-free diet, of bread, butter, cream, peas, peanuts, orange juice, and grape juice, furnishing daily about 1,835 calories, 42 gm. of protein, 0.53 gm. of phosphorus, and 0.28 gm. of calcium.

With Subject K (52 kilos) there was a positive nitrogen balance in all but one of the ten 3 day periods. Approximate equilibrium of phosphorus was established within 3 days and thereafter maintained throughout the month, while the calcium intake proved inadequate for the maintenance of equilibrium even during the second half of the month, the results indicating that the body was unable to adjust itself to a metabolism of less than about 0.35 gm. of calcium, this being the average daily output after the first 12 days.

With L (57 kilos) there was a slight loss of nitrogen during the first half of the month and practical equilibrium during the latter half. The output of phosphorus, especially after the first 6 days, was only slightly greater than the intake, the average for the month being 0.63 gm. per day; or if the first 6 days are regarded as representing a period of adjustment, the average for the rest of the month becomes 0.60 gm. of phosphorus per day. The output of calcium was fairly constant at about 0.30 gm. per day.

In Experiment III, K took a meat-free diet for comparison with the results which she had shown with the mixed diet of

Experiment I. In this last experiment (body weight 50 kilos) there was approximate nitrogen equilibrium on an intake of 6.7 gm.; a steady loss of phosphorus on an intake of 0.57 gm., the output averaging 0.64 gm.; and a steady loss of calcium on an intake of 0.27 gm., the output ranging from 0.27 to 0.42 gm., averaging 0.37 gm. and showing no tendency to approach closer to equilibrium with continuance of the low calcium intake. These experiments therefore indicate for the woman of 50 to 52 kilos (K) a minimum requirement of 0.64 gm. of phosphorus and 0.35 gm. of calcium; for the woman of 57 kilos (L) a requirement of at least 0.60 gm. of phosphorus and 0.31 gm. of calcium. If, for convenience of comparison with experiments upon men, the results are calculated to a basis of 70 kilos on the assumption that the requirement is directly proportional to the body weight, the data would indicate the equivalent of 0.90 and 0.74 gm. of phosphorus, and 0.49 and 0.38 gm. of calcium "per man per day" respectively.

Considering the data of all three experiments there does not appear to be any distinct monthly cycle in the metabolism of either nitrogen, phosphorus, or calcium (except for the previously known tendency to retain nitrogen for a day or so at the beginning of the menstrual period) nor was the output of any one of these three elements in the menstrual flow large enough to affect materially the estimate of the daily requirement for normal metabolism as averaged for the entire month. From this standpoint the menstrual flow is essentially a blood loss and as such is important to the balance of intake and output of iron, but of minor consequence in the nitrogen, phosphorus, or calcium metabolism.

EXPERIMENTAL.

Daily Intake.

Table I shows the kinds and amounts of foods consumed by each subject in each of the three experiments.

In Experiment I the subject took a mixed diet including meat, breadstuffs, fruit, fats, and sugar in normal amounts. It was planned to furnish ample calories and protein while making the intake of phosphorus and calcium low enough to test the requirement for each of these elements.

TABLE I.

Kinds and Amounts of Foods Eaten, with Daily Intake of Calories, Protein, Phosphorus, and Calcium in Each of the Three Experiments.

	Experiment I. Subject K, 52 kg.	Experiment II. Subject L, 57 kg.	Experiment III. Subject K, 50 kg.
	gm.	*gm.*	*gm.*
Meat	139	—	—
Milk	—	—	120
Cream	137	(79)† 105	—
Butter	53	(26)† 39	78
Bread	244	214	234
Shredded wheat	28	—	—
Peas	—	28	—
Peanuts	—	(55)† 36	—
Peanut butter	—	—	50
Apples	—	—	150
Grape juice	—	200	300
Orange juice	232	81	—
Sugar	50	13	50
Cocoa	(3)* —	—	—
Coffee	—	—	100‡
Calories§	2,137	(1,735)† 1,835	2,193
Protein‖	61.90 gm.	42.40 gm.	41.60 gm.
Nitrogen‖	9.90 "	6.80 "	6.70 "
Phosphorus‖	0.70 "	0.53 "	0.57 "
Calcium‖	0.30 "	0.28 "	0.27 "

* First period only.
† " and second periods only.
‡ " three periods only.
§ Calculated from tables by Atwater and Bryant.
‖ By analysis of all articles of food and drink.

In Experiment II the diet contained legumes instead of meat and in addition normal amounts of bread, butter, fruit, and sugar. The energy value and protein content were somewhat lower than in Experiment I, but approximately sufficient for maintenance, while the phosphorus and calcium content was sufficiently below the probable requirement to induce a minimum rate of metabolism of these elements.

In Experiment III the diet was of the same character as in Experiment II except for a somewhat more liberal energy intake the subject being the same as in Experiment I.

Daily Output.

Tables II, III, and IV show the daily output of nitrogen, phosphorus, and calcium in Experiments I, II, and III respectively.

Comparison of Intake and Output.

Table V summarizes the average daily intake and output of nitrogen, phosphorus, and calcium for each period of each experiment. Experiments I and III with K covered 30 and 28 consecutive days, respectively. Experiment II with L covered 30 consecutive days.

TABLE II.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiment I (Subject K).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1915	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	Feb. 17	52.7	7.53	1.01	8.54	0.51	0.25	0.76	0.10	0.28	0.38
	" 18	—	6.47	1.01	7.48	0.54	0.25	0.79	0.11	0.28	0.39
	" 19	51.6	7.18	1.01	8.19	0.56	0.25	0.81	0.11	0.28	0.39
II	" 20	52.3	6.76	1.01	7.77	0.47	0.25	0.72	0.14	0.28	0.42
	" 21	52.0	8.09	1.01	9.10	0.47	0.25	0.72	0.09	0.28	0.37
	" 22	52.1	8.09	1.01	9.10	0.47	0.25	0.72	0.11	0.28	0.39
III	" 23	52.4	7.46	1.13	8.59	0.40	0.22	0.62	0.12	0.27	0.39
	" 24	52.2	7.53	1.13	8.66	0.45	0.22	0.67	0.14	0.27	0.41
	" 25	52.4	7.71	1.13	8.84	0.40	0.22	0.62	0.13	0.27	0.40
IV	" 26	52.4	—	1.74	—	0.51	0.30	0.81	0.12	0.39	0.51
	" 27	—	7.69	1.74	9.43	0.52	0.30	0.82	0.11	0.39	0.50
	" 28	51.4	9.18	1.74	10.92	0.51	0.30	0.81	0.11	0.39	0.50
V	Mar. 1	51.5	7.41	1.38	8.79	0.41	0.25	0.66	0.09	0.29	0.38
	" 2	51.5	7.92	1.38	9.30	0.54	0.25	0.79	0.09	0.29	0.38
	" 3	51.4	7.98	1.38	9.36	0.47	0.25	0.72	0.09	0.29	0.38
VI	" 4	50.9	7.75	0.84	8.59	0.52	0.23	0.75	0.08	0.29	0.37
	" 5	50.9	7.58	0.84	8.42	0.53	0.23	0.76	0.09	0.29	0.38
	" 6	50.9	8.00	0.84	8.84	0.45	0.23	0.68	0.09	0.29	0.38
VII	" 7	50.7	5.83	0.69	7.00†	0.48	0.17	0.66‡	0.08	0.19	0.27§
	" 8	51.2	8.32	0.69	9.49†	0.49	0.17	0.67‡	0.08	0.19	0.27§
	" 9	51.3	7.30	0.69	8.47†	0.53	0.17	0.71‡	0.09	0.19	0.28§
VIII	" 10	51.4	7.36	0.96	8.80†	0.53	0.21	0.73‡	0.09	0.25	0.34§
	" 11	52.0	7.12	0.96	8.08	0.56	0.21	0.78	0.09	0.25	0.34
	" 12	51.2	7.36	0.96	8.32	0.51	0.21	0.72	0.09	0.25	0.34
IX	" 13	51.3	7.29	1.40	8.69	0.48	0.26	0.74	0.10	0.30	0.40
	" 14	51.1	6.99	1.40	8.39	0.42	0.26	0.68	0.07	0.30	0.37
	" 15	51.6	6.78	1.40	8.18	0.54	0.26	0.80	0.09	0.30	0.39
X	" 16	52.0	7.54	1.16	8.70	0.48	0.23	0.71	0.09	0.25	0.34
	" 17	51.7	7.29	1.16	8.45	0.51	0.23	0.74	0.09	0.25	0.34
	" 18	51.8	7.58	1.16	8.74	0.52	0.23	0.75	0.09	0.25	0.34

* The feces belonging to each period were analyzed as a single sample.

† Including 0.48 gm. nitrogen per day for each of these 4 days from menstrual flow.

‡ Including 0.013 gm. phosphorus per day for each of these 4 days from menstrual flow.

§ Including 0.004 gm. calcium per day for each of these 4 days from menstrual flow.

TABLE III.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiment II (Subject L).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1915	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	Feb. 17	57.9	6.96	1.16	8.12	0.39	0.29	0.68	0.09	0.22	0.31
	" 18		6.28	1.16	7.44	0.41	0.29	0.70	0.12	0.22	0.34
	" 19		6.11	1.16	7.27	0.41	0.29	0.70	0.12	0.22	0.34
II	" 20	57.6	7.44	1.43	8.87	0.41	0.31	0.72	0.15	0.21	0.36
	" 21		6.28	1.43	7.71	0.41	0.31	0.72	0.13	0.21	0.34
	" 22		7.53	1.43	8.96	0.38	0.31	0.69	0.13	0.21	0.34
III	" 23	57.0	6.75	1.43	8.18	0.34	0.26	0.60	0.14	0.23	0.37
	" 24		6.46	1.43	7.89	0.33	0.26	0.59	0.14	0.23	0.37
	" 25		6.09	1.43	7.52	0.32	0.26	0.58	0.13	0.23	0.36
IV	" 26		6.60	1.22	7.82	0.36	0.25	0.61	0.13	0.15	0.28
	" 27		6.15	1.22	7.37	0.40	0.25	0.65	0.12	0.15	0.27
	" 28		6.04	1.22	7.26	0.34	0.25	0.59	0.12	0.15	0.27
V	Mar. 1	56.9	6.07	1.46	7.53	0.33	0.26	0.59	0.12	0.22	0.34
	" 2		5.64	1.46	7.10	0.35	0.26	0.61	0.12	0.22	0.34
	" 3		6.71	1.46	8.17	0.36	0.26	0.62	0.12	0.22	0.34
VI	" 4	57.0	5.50	1.35	6.85	0.31	0.26	0.56	0.12	0.16	0.28
	" 5		5.25	1.35	6.60	0.33	0.26	0.59	0.12	0.16	0.28
	" 6		3.76	1.35	5.27†	0.29	0.26	0.55‡	0.08	0.16	0.25§
	" 7		5.45	1.35	6.96†	0.29	0.26	0.55‡	0.13	0.16	0.29§
	" 8		5.11	1.35	6.62†	0.33	0.26	0.59‡	0.14	0.16	0.30§
	" 9		5.76	1.35	7.27†	0.34	0.26	0.60‡	0.15	0.16	0.31§
VII	" 10	56.1	5.08	1.25	6.33	0.40	0.23	0.63	0.15	0.19	0.34
	" 11		5.25	1.25	6.50	0.39	0.23	0.62	0.14	0.19	0.33
	" 12		5.76	1.25	7.01	0.40	0.23	0.63	0.15	0.19	0.34
VIII	" 13	56.0	5.22	1.42	6.64	0.33	0.25	0.58	—	0.21	—
	" 14		5.39	1.42	6.81	0.42	0.25	0.67	0.12	0.21	0.33
	" 15		5.67	1.42	7.09	0.38	0.25	0.63	0.13	0.21	0.34
IX	" 16	56.6	5.22	1.25	6.47	0.40	0.22	0.62	0.10	0.19	0.29
	" 17		5.41	1.25	6.66	0.32	0.22	0.54	0.13	0.19	0.32
	" 18		5.11	1.25	6.36	0.33	0.22	0.55	0.11	0.19	0.30

* The feces belonging to each period were analyzed as a single sample.

† Including 0.164 gm. nitrogen per day for each of these 4 days from menstrual flow.

‡ Including 0.004 gm. phosphorus per day for each of these 4 days from menstrual flow.

§ Including 0.002 gm. calcium per day for each of these 4 days from menstrual flow.

TABLE IV.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiment III (Subject K).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1915	kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	May 27	50.4	6.56	0.79	7.35						
	" 28	50.1	5.54	0.79	6.33	0.47	0.17	0.64	0.07	0.20	0.27
	" 29	49.8	5.68	0.79	6.47						
II	" 30	50.1	5.65	1.10	6.75						
	" 31	50.2	5.60	1.10	6.70						
	June 1	50.4	5.68	1.10	6.78	0.41	0.22	0.63	0.09	0.28	0.37
	" 2	50.3	5.63	1.10	6.73						
III	" 3	50.1	5.69	1.10	6.79						
	" 4	50.3	5.35	1.10	6.45						
	" 5	50.1	6.05	1.10	7.15	0.40	0.22	0.62	0.10	0.28	0.38
	" 6	49.8	4.98	1.10	6.08						
IV	" 7	50.1	5.12	0.98	6.10						
	" 8	50.3	5.43	0.98	6.41						
	" 9	50.2	6.02	0.98	7.00	0.37	0.26	0.63	0.12	0.29	0.41
	" 10	50.1	5.74	0.98	6.72						
V	" 11	50.2	6.24	0.79	7.03						
	" 12	—	5.65	0.79	6.44						
	" 13	50.2	6.30	0.79	7.09	0.39	0.24	0.63	0.12	0.27	0.39
	" 14	49.9	6.13	0.79	6.92						
VI	" 15	50.1	5.76	0.93	6.69						
	" 16	50.0	5.92	0.93	6.85						
	" 17	49.5	5.15	0.93	6.08	0.38	0.26	0.64	0.12	0.26	0.38
	" 18	49.6	5.69	0.93	6.62						
VII	" 19	49.8	5.63	0.89	6.83†						
	" 20	—	5.69	0.89	6.89†						
	" 21	49.6	6.16	0.89	7.36†	0.42	0.28	0.71‡	0.12	0.29	0.41§
	" 22	49.7	5.72	0.89	6.92†						
	" 23	49.7	5.98	0.89	7.18†						

* The feces belonging to each period were analyzed as a single sample.

† Including 0.31 gm. nitrogen per day for each of these 5 days from menstrual flow.

‡ Including 0.006 gm. phosphorus per day for each of these 5 days from menstrual flow.

§ Including 0.004 gm. calcium per day for each of these 5 days from menstrual flow.

TABLE V.

Summary of Daily Intake and Output of Nitrogen, Phosphorus, and Calcium.

	Experimental period.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.	Intake.	Output.	Balance.
		kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Experiment I. Subject K, 3 day periods.	I	52.0	10.17	8.07	+2.10	0.69	0.79	−0.10	0.30	0.39	−0.09
	II	52.1	10.05	8.66	+1.39	0.68	0.72	−0.04	0.30	0.39	−0.09
	III	52.3	10.04	8.70	+1.34	0.73	0.64	+0.09	0.29	0.40	−0.11
	IV	51.9	9.77	10.27	−0.50	0.63	0.81	−0.18	0.29	0.50	−0.21
	V	51.5	9.96	9.15	+0.81	0.70	0.72	−0.02	0.29	0.38	−0.09
	VI	50.9	9.59	8.62	+0.97	0.67	0.73	−0.06	0.29	0.38	−0.09
	VII*	51.1	9.44	8.32	+1.12	0.67	0.68	−0.01	0.30	0.28	+0.02
	VIII*	51.5	9.90	8.40	+1.50	0.71	0.74	−0.03	0.30	0.34	−0.04
	IX	51.3	10.34	8.42	+1.92	0.75	0.74	+0.01	0.30	0.39	−0.09
	X	51.8	9.88	8.63	+1.25	0.73	0.73	±0.00	0.30	0.34	−0.04
	Average	51.6	9.91	8.72	+1.19	0.70	0.73	−0.03	0.30	0.38	−0.08
Experiment II. Subject L, 3 day periods.	I	57.9	7.26	7.61	−0.35	0.56	0.69	−0.13	0.27	0.33	−0.06
	II	57.6	7.15	8.51	−1.36	0.55	0.71	−0.16	0.27	0.35	−0.08
	III	57.0	6.77	7.86	−1.09	0.56	0.59	−0.03	0.28	0.37	−0.09
	IV	—	6.59	7.48	−0.89	0.48	0.62	−0.14	0.28	0.27	+0.01
	V	57.0	6.72	7.60	−0.88	0.53	0.61	−0.08	0.28	0.34	−0.06
	VI*†	56.6	6.38	6.26	+0.12	0.51	0.58	−0.07	0.28	0.28	±0.00
	VII	—	6.61	6.61	±0.00	0.52	0.63	−0.11	0.28	0.33	−0.05
	VIII	56.0	6.98	6.85	+0.13	0.55	0.63	−0.08	0.28	0.34	−0.06
	IX	56.6	6.59	6.50	+0.09	0.53	0.57	−0.04	0.28	0.30	−0.02
	Average	57.0	6.78	7.25	−0.47	0.53	0.63	−0.10	0.28	0.32	−0.04
Experiment III. Subject K, 4 day periods.	I	50.1	6.68	6.72	−0.04	0.57	0.64	−0.07	0.27	0.27	±0.00
	II	50.3	6.76	6.74	+0.02	0.57	0.63	−0.06	0.27	0.37	−0.10
	III	50.1	6.73	6.62	+0.11	0.57	0.62	−0.05	0.28	0.38	−0.10
	IV	50.2	6.47	6.56	−0.09	0.56	0.63	−0.07	0.27	0.41	−0.14
	V	50.1	6.69	6.87	−0.18	0.56	0.63	−0.07	0.27	0.39	−0.12
	VI	49.7	6.57	6.56	+0.01	0.57	0.64	−0.07	0.27	0.38	−0.11
	VII*	49.7	6.63	7.03	−0.40	0.56	0.71	−0.15	0.28	0.41	−0.13
	Average	50.0	6.65	6.74	−0.09	0.57	0.64	−0.07	0.27	0.37	−0.10

* Menstrual period.

† 6 day period.

EXPERIMENTS ON THE NUTRITIVE VALUE OF MAIZE PROTEIN AND ON THE PHOSPHORUS AND CALCIUM REQUIREMENTS OF HEALTHY WOMEN.*

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The experiments described in this paper were designed to continue the study of the phosphorus and calcium requirements of healthy women, a part of which has been recorded in an earlier communication from this laboratory[1] and to include also a preliminary study of the efficiency of utilization of maize products in human nutrition.

Two experiments each including several periods (or two series of consecutive experiments) were performed with each of two young women, Subjects M and N. In the first series a uniform diet consisting of wheat bread, butter, peanut butter, milk, meat, apples, and grape juice was followed for 28 days and the nitrogen, phosphorus, and calcium balances were determined in seven consecutive periods of 4 days each.

In the second series, diets containing corn meal were used. Subject M took for 23 consecutive days a uniform diet which included 200 gm. of corn meal per day, about one-third of the protein being thus derived from maize. Subject N in the second series took for 20 days a diet consisting largely of wheat bread and then for 8 days substituted corn meal for a part of the wheat flour used in making the bread and also for part of the sugar pre-

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[1] Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373.

viously used, about one-fifth of the protein of the diet of these 8 days being derived from maize.

The experience of these subjects indicated that without the use of eggs or of a liberal amount of milk in cooking, it is difficult for a person not previously accustomed to eating maize to take more than about one-fifth of the food protein in this form constantly day after day, together with an equal or greater amount of wheat flour, without unfavorable influence upon appetite or digestion.[2] The use by M of 200 gm. of corn meal daily, enough to furnish one-quarter to one-third of the calories and protein of the diet, was attended by nitrogen equilibrium on a protein intake of 46 gm. (body weight 59 kilos) showing that the diet as a whole was efficiently used in the protein metabolism; but its use for 3 weeks led to digestive disturbance which the subject felt was directly attributable to the continued consumption of this large proportion of corn meal. In this connection, however, it must be noted that the experimental conditions were unusually severe in that the amount of corn meal used each day was about equal to the usual entire consumption of breadstuffs and other cereal products, and along with this was taken about an equal amount of wheat flour, so that there was an undue preponderance of bread and mush in the diet with no meat or eggs, and only very limited amounts of milk, fruits, or vegetables.

Considering the nature and circumstances of the experiment, the results must be regarded as highly favorable to the efficiency of maize protein in normal (adult) human nutrition, (1) because the continued use of a low protein diet, in which 47 per cent of the total protein was from wheat flour and 31 per cent from corn meal, showed the latter to be efficiently used in the maintenance of nitrogen equilibrium, and (2) because the abrupt substitution of maize protein for wheat protein to an extent affecting one-fifth of the total protein intake was without any unfavorable effect upon the nitrogen balance.

As regards the evidence of these experiments upon phosphorus requirement, M (weighing 57 and 60 kilos) showed after the first

[2] In a later experiment by another subject, a much larger amount of corn meal was eaten without disturbance of appetite or digestion; but in this case it was cooked at a higher temperature and the diet contained no other starchy food.

3 days of the first series, a fairly regular output averaging 0.73 gm. of phosphorus per day, which would appear to represent her minimum requirement under the conditions of this experiment inasmuch as it involved a continued minus balance which the body was apparently unable to bring to equilibrium by effecting even a small decrease in its rate of phosphorus metabolism. In the second series the output for this subject was almost the same as in the first series, *viz.*, 0.71 gm. of phosphorus per day. Subject N (weighing 54 and 55 kilos) showed in the first series a minimum output for a single 4 day period of 0.69 gm. of phosphorus per day with an average of 0.78 gm. In the second series which was less well adapted to test the actual requirement of the phosphorus metabolism she showed during the last period of 4 days a minimum output of 0.70 gm. of phosphorus per day. The metabolism of 0.71 and 0.69 gm. of phosphorus per day as minima for these two women weighing 60 and 54 kilos respectively, would, if computed on the assumption that the requirement is proportional to the body weight, correspond to 0.83 and 0.89 gm. respectively as the minimum requirement of a man of average size (70 kilos) per day. This is in satisfactory agreement with the corresponding data for the two women previously studied which were 0.91 and 0.72 gm. of phosphorus per 70 kilos of body weight respectively.

The calcium metabolism was studied in only the first series of experiments with each subject. Subject M showed after the first 3 days an average output of 0.24 gm. of calcium per day involving a persistent negative balance with no tendency toward equilibrium. This therefore probably represents her minimum rate of calcium output and somewhat less than the minimum amount on which equilibrium could have been established. Subject N showed an average daily output of 0.34 gm. with persistent minus balance and no tendency to equilibrium. The two subjects previously studied weighing 50 and 58 kilos respectively showed calcium minima of 0.35 and 0.31 gm. per day. If the data for the four women (Subjects K, L, M, N) are calculated to a uniform basis of 70 kilos of body weight they become equivalent to 0.49, 0.38, 0.29, and 0.44 gm. of calcium "per man per day" respectively. Relative to the actual amounts involved these figures would superficially appear to show a distinctly larger

individual variation in the calcium than in the phosphorus requirement, but as noted above the lowest figure for calcium may very likely be less than the minimum amount on which this subject could have established equilibrium. For the three subjects in whom there was approximate equilibrium, the individual variations are not large, the calcium output being 0.49, 0.38, and 0.44 gm. per 70 kilos of body weight per day, respectively, and the average is substantially identical with the average of all the data on calcium requirement in normal human nutrition which we have found. Although the data reported in 63 individual experiments range from 0.27 to 0.78 gm., a range so wide as to throw doubt upon the validity of the average, yet since the average of all these experiments and the results of the three extended studies here referred to are in such close agreement, it seems probable that this average of 0.45 gm. of calcium per 70 kilos of body weight per day does approximately represent the minimum maintenance requirement for normal human nutrition.

EXPERIMENTAL.

Tables I to VII, which follow, give in condensed form the quantitative data of intake and output for the four experiments herein described.

TABLE I.

Kinds and Amounts of Foods Eaten, with Daily Intake of Calories, Protein, Phosphorus, and Calcium.

Kind of food.	Experiment I. Subject M, 57 kg.	Experiment II. Subject N, 54 kg.	Experiment III. Subject M, 59 kg.	Experiment IV. Periods I–V. Subject N, 55 kg.	Experiment IV. Periods VI–VII. Subject N, 55 kg.
	gm.	gm.	gm.	gm.	gm.
Meat	150	150	—	—	—
Milk	—	120	300	300	300
Butter	100	80	80	80	80
Olive oil	—	—	10	11	11
Bread	250	250	—	370*	—
Wheat flour	—	—	220	125‡	—
" " entire	—	—	—	175‡	220
Corn meal	—	—	200	—	110
Sugar	25	25	30	50	25
Lettuce	—	—	35	35	35
Apple	125	105	200	200	200
Grape juice	300	150	—	—	—
Peanut butter	30	20	—	—	—
Yeast	—	—	10	10	10
Coffee	—	200†		—	—
Calories§	2,294	2,017	2,664	2,363	2,345
Protein‖	64.3 gm.	64.3 gm.	46.9 gm.	44.4 gm.	41.2 gm.
Nitrogen‖	10.3 "	10.3 "	7.5 "	7.1 "	6.6 "
Phosphorus‖	0.68 "	0.73 "	0.59 "	1.00 "	0.60 "
Calcium	0.14 " ‖	0.25 " ‖	0.48 " §	0.46 " §	0.44 " §

* Period I only.

† Periods I, II, and III only.

‡ Periods II–V.

§ Calculated from the revised tables of Sherman.[3]

‖ By analysis (protein = nitrogen × 6.25).

[3] Sherman, H. C., Chemistry of Food and Nutrition, New York, 2nd edition, 1918.

TABLE II.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiment I (Subject M).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1915	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	May 26	57.1	6.89	1.02	7.91	0.59	0.21	0.80	0.08	0.20	0.28
	" 27	57.0	10.62	1.02	11.64	0.59	0.21	0.80	0.08	0.20	0.28
	" 28	56.4	9.90	1.02	10.92	0.59	0.21	0.80	0.08	0.20	0.28
	" 29	56.4	8.80	1.02	9.82	0.59	0.21	0.80	0.08	0.20	0.28
II	" 30	56.6	9.20	0.81	10.01	0.54	0.15	0.69	0.11	0.12	0.23
	" 31	56.9	8.58	0.81	9.39	0.54	0.15	0.69	0.11	0.12	0.23
	June 1	57.3	9.40	0.81	10.21	0.54	0.15	0.69	0.11	0.12	0.23
	" 2	56.9	9.37	0.81	10.18	0.54	0.15	0.69	0.11	0.12	0.23
III	" 3	57.0	8.97	1.11	10.08	0.60	0.17	0.77	0.12	0.12	0.24
	" 4	57.0	9.39	1.11	10.50	0.60	0.17	0.77	0.12	0.12	0.24
	" 5	57.1	9.42	1.11	10.53	0.60	0.17	0.77	0.12	0.12	0.24
	" 6	56.7	8.72	1.11	9.83	0.60	0.17	0.77	0.12	0.12	0.24
IV	" 7	56.7	9.50	0.82	11.13†	0.60	0.13	0.74‡	0.13	0.09	0.22§
	" 8	56.9	8.66	0.82	10.29†	0.60	0.13	0.74‡	0.13	0.09	0.22§
	" 9	56.8	9.34	0.82	10.97†	0.60	0.13	0.74‡	0.13	0.09	0.22§
	" 10	56.6	9.42	0.82	11.05†	0.60	0.13	0.74‡	0.13	0.09	0.22§
V	" 11	57.0	10.01	0.89	10.90	0.57	0.17	0.74	0.15	0.12	0.27
	" 12	56.6	8.89	0.89	9.78	0.57	0.17	0.74	0.15	0.12	0.27
	" 13	55.9	9.59	0.89	10.48	0.57	0.17	0.74	0.15	0.12	0.27
	" 14	56.6	8.33	0.89	9.22	0.57	0.17	0.74	0.15	0.12	0.27
VI	" 15	57.1	9.42	1.29	10.71	0.49	0.23	0.72	0.13	0.15	0.28
	" 16	56.8	8.76	1.29	10.05	0.49	0.23	0.72	0.13	0.15	0.28
	" 17	57.3	8.08	1.29	9.37	0.49	0.23	0.72	0.13	0.15	0.28
	" 18	57.2	9.28	1.29	10.57	0.49	0.23	0.72	0.13	0.15	0.28
VII	" 19	57.5	9.76	0.78	10.54	0.57	0.17	0.74	0.12	0.11	0.23
	" 20	56.7	10.04	0.78	10.82	0.57	0.17	0.74	0.12	0.11	0.23
	" 21	56.7	10.22	0.78	11.00	0.57	0.17	0.74	0.12	0.11	0.23
	" 22	56.5	9.68	0.78	10.46	0.57	0.17	0.74	0.12	0.11	0.23

* The feces belonging to each period were analyzed as a single sample. In this experiment the same is true of the determinations of phosphorus and calcium in the urine.

† Including 0.81 gm. nitrogen for each of 4 days from menstrual flow.
‡ " 0.013 " phosphorus " " " 4 " " " "
§ " 0.0014 " calcium " " " 4 " " " "

TABLE III.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiment II (Subject N).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1915	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	May 26	54.8	8.34	1.66	10.63†	0.52	0.29	0.82‡	0.10	0.25	0.35§
	" 27	54.2	9.45	1.66	11.74†	0.52	0.29	0.82‡	0.10	0.25	0.35§
	" 28	54.0	7.43	1.66	9.72†	0.52	0.29	0.82‡	0.10	0.25	0.35§
	" 29	53.9	8.27	1.66	10.56†	0.52	0.29	0.82‡	0.10	0.25	0.35§
II	" 30	54.9	8.69	1.24	10.56†	0.56	0.21	0.78‡	0.13	0.19	0.33§
	" 31	54.8	8.10	1.24	9.34	0.56	0.21	0.77	0.13	0.19	0.32
	June 1	55.2	—	1.24	—	0.56	0.21	0.77	0.13	0.19	0.32
	" 2	55.2	9.20	1.24	10.44	0.56	0.21	0.77	0.13	0.19	0.32
III	" 3	54.6	8.44	1.11	9.55	0.58	0.20	0.78	0.14	0.18	0.32
	" 4	54.5	9.59	1.11	10.70	0.58	0.20	0.78	0.14	0.18	0.32
	" 5	54.3	9.79	1.11	10.90	0.58	0.20	0.78	0.14	0.18	0.32
	" 6	54.1	9.08	1.11	10.19	0.58	0.20	0.78	0.14	0.18	0.32
IV	" 7	54.4	9.84	1.05	10.89	0.59	0.21	0.80	0.13	0.19	0.32
	" 8	54.4	8.83	1.05	9.88	0.59	0.21	0.80	0.13	0.19	0.32
	" 9	54.6	9.42	1.05	10.47	0.59	0.21	0.80	0.13	0.19	0.32
	" 10	54.3	8.78	1.05	9.83	0.59	0.21	0.80	0.13	0.19	0.32
V	" 11	54.2	8.97	1.05	10.02	0.51	0.18	0.69	0.18	0.18	0.36
	" 12	54.6	7.93	1.05	8.98	0.51	0.18	0.69	0.18	0.18	0.36
	" 13	54.3	7.60	1.05	8.65	0.51	0.18	0.69	0.18	0.18	0.36
	" 14	54.1	8.64	1.05	9.69	0.51	0.18	0.69	0.18	0.18	0.36
VI	" 15	54.7	9.82	1.12	10.94	0.62	0.21	0.83	0.19	0.19	0.38
	" 16	55.0	10.10	1.12	11.22	0.62	0.21	0.83	0.19	0.19	0.38
	" 17	54.5	10.36	1.12	11.48	0.62	0.21	0.83	0.19	0.19	0.38
	" 18	54.3	10.04	1.12	11.16	0.62	0.21	0.83	0.19	0.19	0.38
VII	" 19	53.6	9.82	1.18	11.00	0.60	0.22	0.82	0.17	0.20	0.37
	" 20	—	10.36	1.18	11.54	0.60	0.22	0.82	0.17	0.20	0.37
	" 21	53.7	9.11	1.18	10.29	0.60	0.22	0.82	0.17	0.20	0.37
	" 22	53.1	10.04	1.18	11.22	0.60	0.22	0.82	0.17	0.20	0.37

* The feces belonging to each period were analyzed as a single sample. In this experiment the same is true of the determinations of phosphorus and calcium in the urine.

† Including 0.63 gm. nitrogen for each of 5 days from menstrual flow.

‡ " 0.009 " phosphorus " " " 5 " " " "

§ " 0.002 " calcium " " " 5 " " " "

TABLE IV.

Daily Output of Nitrogen and Phosphorus in Experiment III (Subject M).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1917	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	July 4	59.0†	7.32	0.73	8.05	0.52	0.21	0.73
	" 5	59.4	5.90	0.73	6.63	0.52	0.21	0.73
	" 6	58.9	6.31	0.73	7.04	0.52	0.21	0.73
II	" 7	59.3	5.83	0.85	6.68	0.53	0.21	0.74
	" 8	59.3	7.44	0.85	8.29	0.53	0.21	0.74
	" 9	59.6	6.53	0.85	8.22‡	0.53	0.21	0.75§
	" 10	59.3	6.24	0.85	7.93‡	0.53	0.21	0.76§
III	" 11	59.3	6.38	1.04	8.26‡	0.42	0.24	0.68§
	" 12	59.5	6.06	1.04	7.94‡	0.42	0.24	0.67§
	" 13	59.8	6.08	1.04	7.12	0.42	0.24	0.66
	" 14	59.9	6.72	1.04	7.76	0.42	0.24	0.66
IV	" 15	59.9	6.30	0.70	7.00	0.50	0.16	0.66
	" 16	59.9	6.52	0.70	7.22	0.50	0.16	0.66
	" 17	59.9	6.52	0.70	7.22	0.50	0.16	0.66
	" 18	60.2	5.70	0.70	6.40	0.50	0.16	0.66
V	" 19	59.9	5.72	1.08	6.80	0.43	0.27	0.70
	" 20	59.9	6.43	1.08	7.51	0.43	0.27	0.70
	" 21	60.2	6.58	1.08	7.66	0.43	0.27	0.70
	" 22	59.9	6.02	1.08	7.10	0.43	0.27	0.70
VI	" 23	59.9	6.21	1.08	7.29	0.48	0.27	0.75
	" 24	59.9	5.87	1.08	6.95	0.48	0.27	0.75
	" 25	59.6	6.75	1.08	7.83	0.48	0.27	0.75
	" 26	59.4	6.22	1.08	7.30	0.48	0.27	0.75

* The feces belonging to each period were analyzed as a single sample. In this experiment the same is true of the determination of phosphorus in the urine.

† Weights given include summer clothing which weighed approximately 1.5 kilos

‡ Including 0.84 gm. nitrogen for each of 4 days from menstrual flow.

§ " 0.015 " phosphorus " " " 4 " " " "

TABLE V.

Daily Output of Nitrogen and Phosphorus in Experiment IV (Subject N).

Experimental period.	Day.	Body weight.	Nitrogen.			Phosphorus.		
			Urine.	Feces.*	Total.	Urine.	Feces.*	Total.
	1917	kg.	gm.	gm.	gm.	gm.	gm.	gm.
I	July 5	55.2	5.63	0.54	6.17	0.60	0.14	0.74
	" 6	55.1	4.44	0.54	4.98	0.60	0.14	0.74
II	" 7	54.7	5.99	1.18	7.17	0.72	0.50	1.22
	" 8	54.9	6.16	1.18	7.34	0.72	0.50	1.22
	" 9	55.1	5.50	1.18	6.68	0.72	0.50	1.22
	" 10	55.4	6.55	1.18	7.73	0.72	0.50	1.22
III	" 11	55.2	6.12	1.40	7.52	0.71	0.56	1.27
	" 12	55.1	6.18	1.40	7.58	0.71	0.56	1.27
	" 13	54.9	5.80	1.40	7.20	0.71	0.56	1.27
	" 14	55.1	5.82	1.40	7.22	0.71	0.56	1.27
IV	" 15	55.2	6.32	1.16	7.48	0.62	0.58	1.20
	" 16	55.2	6.77	1.16	7.93	0.62	0.58	1.20
	" 17	55.0	6.55	1.16	7.71	0.62	0.58	1.20
	" 18	54.7	6.27	1.16	7.43	0.62	0.58	1.20
V	" 19	54.9	6.38	1.22	7.60	0.57	0.56	1.13
	" 20	54.8	6.11	1.22	7.33	0.57	0.56	1.13
	" 21	54.7	6.20	1.22	7.42	0.57	0.56	1.13
	" 22	55.0	6.32	1.22	7.54	0.57	0.56	1.13
VI	" 23	54.7	6.07	0.96	7.03	0.49	0.26	0.75
	" 24	54.8	5.87	0.96	6.83	0.49	0.26	0.75
	" 25	54.9	5.36	0.96	6.32	0.49	0.26	0.75
	" 26	55.0	5.29	0.96	6.62†	0.49	0.26	0.76‡
VII	" 27	55.4	5.38	0.89	6.64†	0.48	0.21	0.70‡
	" 28	55.4	5.82	0.89	7.08†	0.48	0.21	0.70‡
	" 29	55.1	5.34	0.89	6.60†	0.48	0.21	0.70‡
	" 30	55.0	5.11	0.89	6.37†	0.48	0.21	0.70‡

* The feces be onging to each period were analyzed as a single sample. In this experiment the same is true of the determination of phosphorus in the urin·.

† Including 0.37 gm. nitrogen for each of 5 days from menstrual flow.

‡ " 0.006 " phosphorus " " " 5 " " " " "

TABLE VI.

Summary of Daily Intake and Output of Nitrogen, Phosphorus, and Calcium in Experiments I and II.

	Period.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.	Intake.	Output.	Balance.
		kg.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Experiment I.	I	56.7	10.30	10.07	+0.23	0.68	0.80	−0.12	0.14	0.28	−0.14
Subject M, 4	II	56.9	10.32	9.95	+0.37	0.67	0.69	−0.02	0.15	0.23	−0.08
day periods.	III	57.0	10.36	10.26	+0.10	0.69	0.77	−0.08	0.14	0.24	−0.10
	IV	56.8	10.15	10.86	−0.71	0.65	0.74	−0.09	0.14	0.22	−0.08
	V	56.5	10.30	10.10	+0.20	0.68	0.74	−0.06	0.13	0.27	−0.14
	VI	57.1	10.34	10.18	+0.16	0.69	0.72	−0.03	0.13	0.28	−0.15
	VII	56.9	10.19	10.70	−0.51	0.68	0.74	−0.06	0.13	0.23	−0.10
Average.....		56.8	10.28	10.30	−0.02	0.68	0.74	−0.06	0.14	0.25	−0.11
Experiment II.	I	54.2	10.33	10.66	−0.33	0.74	0.82	−0.08	0.25	0.35	−0.10
Subject N, 4	II	55.0	10.36	10.11	+0.25	0.73	0.78	−0.05	0.26	0.33	−0.07
day periods.	III	54.4	10.41	10.34	+0.07	0.75	0.78	−0.03	0.26	0.32	−0.06
	IV	54.4	10.12	10.27	−0.15	0.70	0.80	−0.10	0.25	0.33	−0.08
	V	54.3	10.28	9.33	+0.95	0.74	0.69	+0.05	0.25	0.36	−0.11
	VI	54.6	10.33	11.20	−0.87	0.73	0.83	−0.10	0.25	0.38	−0.13
	VII	53.4	10.17	11.01	−0.84	0.73	0.82	−0.09	0.25	0.36	−0.11
Average.....		54.3	10.29	10.42	−0.13	0.73	0.79	−0.06	0.25	0.35	−0.10

TABLE VII.

Summary of Daily Intake and Output of Nitrogen and Phosphorus in Experiments III and IV.

	Period.	Body weight.	Nitrogen.			Phosphorus.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.
		kg.	gm.	gm.	gm.	gm.	gm.	gm.
Experiment III.	I*	59.1†	7.26	7.24	+0.02	0.52	0.73	−0.21
Subject M, 4 day periods.	II	59.4†	7.61	7.78	−0.17	0.60	0.75	−0.15
	III	59.6†	7.65	7.77	−0.12	0.60	0.67	−0.07
	IV	60.0†	7.63	6.96	+0.67	0.61	0.66	−0.05
	V	60.0†	7.48	7.27	+0.21	0.61	0.70	−0.09
	VI	59.7†	7.30	7.34	−0.04	0.61	0.75	−0.14
Average		59.6†	7.49	7.39	+0.10	0.59	0.71	−0.12
Same for Periods IV–VI			7.47	7.19	+0.28	0.61	0.70	−0.09
Experiment IV.	I‡	55.2	7.86	5.57	+2.29	0.57	0.74	−0.17
Subject N, 4 day periods.	II	55.0	6.93	7.23	−0.30	1.10	1.22	−0.12
	III	55.1	6.97	7.38	−0.41	1.10	1.27	−0.17
	IV	55.0	6.96	7.64	−0.68	1.10	1.20	−0.10
	V	54.9	6.89	7.47	−0.58	1.11	1.13	−0.02
	VI	54.9	6.58	6.70	−0.12	0.60	0.75	−0.15
	VII	55.2	6.57	6.67	−0.10	0.61	0.70	−0.09
Average		55.0	6.97	6.94	+0.03	0.88	1.00	−0.12
Same for Periods VI and VII			6.58	6.69	−0.11	0.61	0.73	−0.12

* 3 day period only. All other periods were 4 days each.

† Weighed in summer clothing which was approximately constant at 1.5 kilos.

‡ 2 day period only. All other periods were of 4 days each.

THE INFLUENCE OF NEUTRAL SALTS UPON THE VISCOSITY OF GELATIN SOLUTIONS.

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I.

When dried pig's bladder or powdered gelatin is treated with a neutral salt a certain swelling occurs; after the salt is washed away a much greater additional swelling is noticed when the cation of the neutral salt used was monovalent. When the cation of the salt used was bivalent no such additional swelling occurs.[1] These phenomena had been explained by the writer on the assumption that neutral salts with monovalent cation (Li, Na, K, NH_4) form with gelatin and certain other proteins highly ionizable metal proteinates which dissociate electrolytically into a positive metal ion (that of the salt used) and a negative protein (gelatin) ion. It is the latter which is responsible for the additional swelling after the excess of the salt is washed away, the ionization of the metal gelatinate being repressed in the presence of the excess of salt. The salts with bivalent cation (Mg, Ca, Sr, Ba) were assumed to form non- or less ionizable metal gelatinates and hence no or little additional swelling of gelatin was observed after the excess of neutral salts of bivalent cations was washed away.[2,3]

The new experiments reported in this note were undertaken with the intention of testing the idea that the difference in the influence of the monovalent and bivalent cations upon the additional swelling is due to a difference in the degree of ionization of the metal gelatinates with monovalent and bivalent cations.

[1] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.
[2] Loeb, *J. Biol. Chem.*, 1918, xxxiii, 531.
[3] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77.

It has been pointed out by various authors—Laqueur and Sackur,[4] Hardy,[5] Pauli,[6] Bottazzi,[7] and Robertson[8]—that solutions of ionized protein have a much higher viscosity than solutions of the same protein in non-ionized condition. These statements are based on experiments with acids and bases, but not with neutral salts. If they are correct—as they seem to be—then the measurements of viscosity should offer a means of testing our hypothesis that neutral salts with univalent metal (type NaCl and Na_2SO_4) react with gelatin under formation of highly ionizable metal gelatinates (*e.g.* sodium gelatinate), while neutral salts with bivalent metal (type $CaCl_2$) form metal gelatinates (*e.g.* calcium gelatinate) which are not or only slightly capable of ionization.

II.

The method of our procedure was as follows. 1 gm. of finely powdered gelatin was put into 100 cc. of a neutral salt solution, whose concentration was generally M/8, and left in this solution for about 1 hour. The powder was stirred repeatedly in the solution. It was then poured into one of the cylindrical funnels described in the previous papers and the salt solution was allowed to filter off as much as possible. To remove the last traces of salt solution the gelatin mass was then perfused four times with 25 cc. of H_2O. Tests with silver nitrate showed that the water filtering off in the fourth perfusion no longer gave a precipitate when the salt used was a chloride. The removal of the salt solution is necessary since its presence represses the ionization of the metal gelatinate formed. It was the neglect of this precaution which led to the erroneous results of some previous experimenters on the action of neutral salts on proteins. Hence our viscosity measurements were all made with gelatin freed from the excess of the salt (with which it had been treated) *and dissolved*

[4] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[5] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

[6] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

[7] Bottazzi, F., *Atti. Accad. Lincei*, 1913, xxii, pt. 2, 141, 263.

[8] Robertson, T. B., The Physical Chemistry of the Proteins, New York, 1918, 320.

in distilled water (and not in the salt solution with which the gelatin had previously been treated).

It is well known that the viscosity of gelatin solution increases with standing even if the solution remains liquid. This has been ascribed to the fact that the gelatin solution has a similar structure to the solid gelatin,[8] but that this structure is only gradually reached by the liquid. We may say that gelatin solution upon standing changes towards a gel structure though it may never reach the actual gel stage. Since we now know through the work of Langmuir that molecules are not spherical, but that we may have to conceive them in the case of proteins as having a chain structure, we may assume that the steady increase of viscosity of gelatin solution upon standing is due to a gradual orientation of gelatin molecules or ions towards the arrangement they possess in a solid gel. On that assumption we understand also the influence of temperature upon the viscosity of gelatin solutions.

Reproducible measurements of viscosity of gelatin solutions can only be obtained when these two chief influences, time and temperature, are kept equal. Our mode of procedure was as follows.

After the mass of gelatin had been washed free from the salt solution with which it had been treated it was liquefied by heating in a water bath at about 50°C. for about 10 minutes. The solution containing 1 gm. of the dry gelatin was then made up to a volume of 100 cc. by adding the necessary amount of *distilled water* and the temperature of the solution lowered to 24°C. The viscosity was determined *immediately* at that temperature, the Ostwald viscometer being in a water bath of 24°C.

In this way we got comparatively constant and reproducible results. The slight variations observed for the same solution were due to the fact that in some cases the gelatin was liquefied immediately after perfusion while in other experiments we waited until the next morning. In the latter case the viscosity was always lower, possibly on account of some hydrolysis or putrefaction.

The time of outflow of distilled water through our viscometer was 53 seconds at 24°C.

A 1 per cent gelatin solution made up of powdered gelatin washed repeatedly with distilled water gave in different experi-

ments the following times of outflow: 84, 83, 78, 84, 86, 80, 83, 76, 83, 82, 83, and 84 seconds, averaging 82 seconds. We may therefore say, a 1 per cent solution of the gelatin used by us (possessing a negligible degree of electrolytic dissociation) has a viscosity corresponding to a time of outflow of 82 seconds through our viscometer.

It seemed advisable to determine the influence of the concentration of the salt solution used upon the result. For this purpose 1 gm. of finely powdered Cooper's non-bleached gelatin was put for 1 hour into each of a series of beakers containing 100 cc. of a NaCl solution varying from M/2 to M/1024 in concentration. After this the gelatin from each solution was put into a separate cylindrical funnel, drained off, and perfused four times with distilled water.[9] The water running off in the fourth perfusion was practically free from chloride when the gelatin had been treated with an M/8 solution of NaCl. The gelatin was therefore free from salt except that held in chemical combination. Three effects of the neutral salts upon the gelatin were measured, namely:

1. The additional swelling; *i.e.*, the increase in swelling which occurred in the cylindrical column of gelatin after the salt was washed away (and the ionization was no longer repressed). The additional swelling was measured in mm. of the height of the column of gelatin in the cylindrical funnels which all had the same diameter.

2. The viscosity of the washed gelatin. The viscosity was expected to increase parallel with the additional swelling, if both additional swelling and increase in viscosity were due to the same factor; namely, increased ionization of the gelatin. The viscosity measurements are given in times (seconds) of outflow through the viscometer.

3. The amount of 95 per cent alcohol required to precipitate 5 cc. of a 1 per cent gelatin solution (washed) and made up in distilled water. According to Pauli, ionized protein loses its precipitability by alcohol. Hence when the ionization of the gelatin exceeds a certain limit it should cease to be precipitable by alcohol. We assumed that this limit was reached when 25 cc.

[9] Those treated with M/2 and M/4 NaCl were perfused six times with 25 cc. of distilled water.

or more of alcohol no longer gave a precipitate and we indicate this by the symbol ∞.

The reader will notice that in all cases the measurements were made on gelatin in the *absence* of the salt solution with which it had been treated. As has been stated in this and the previous paper, the measurements in the presence of salts are of restricted value, since the ionizing effect of the salt on the protein is concealed in the presence of the salt.

Table I gives the three groups of measurements.

TABLE I.

1 gm. gelatin treated for 1 hour with the following solutions of NaCl and then freed from the supernatant solution by four perfusions with 25 cc. of distilled water.

	M 2	M 4	M 8	M 16	M 32	M 64	M 128	M 256	M 512	M 1024	H_2O (control)
Additional swelling in mm. of the height of the cylindrical column of gelatin..	43	40	41	38	40	30	24	20	9	9	5.5
Viscosity of 1 per cent gelatin solution in H_2O in seconds of time of outflow.....	99	99	98	97	95	93	90	89	87	86	85
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H_2O.....	∞	∞	∞	∞	∞	∞	11.3	6.6	5.7	5.7	5.5

These measurements show that beyond a concentration of M/8 a further increase of concentration does not influence the results materially. We therefore used salts with univalent ions in this concentration, while salts with bivalent ions were generally though not always used in M/16 concentration.

III.

We now proceeded to test our hypotheses, namely:

1. That neutral salts with univalent cation (type NaCl and Na_2SO_4) form with common gelatin highly ionizable gelatin compounds (metal gelatinates).

2. That neutral salts of the alkali earth metals (type $CaCl_2$) form compounds with gelatin (metal gelatinates) which are only slightly dissociable.

If these hypotheses are correct, we should expect, first, that gelatin treated for 1 hour with an M/8 solution of any neutral salt with univalent cation (Li, Na, K, NH_4) should (when freed from the excess of salt by perfusion with H_2O) have a considerably increased viscosity; *i.e.*, a time of outflow considerably higher than 82 seconds. Moreover, the time of outflow should be exactly the same for all the salts used regardless of the nature of the cation as long as this is univalent, and regardless of the nature of the anion, whether Cl, NO_3, acetate, sulfate, tartrate, succinate, etc.; the only provision being that the salt solution originally applied should always have the same concentration of the univalent cation (M/8 in our experiments). All this turned out as our theory demanded. In our experiments 1 gm. of powdered gelatin was allowed to interact for 1 hour with an M/8 solution of salts of the type NaCl and with M/16 solutions of salts of the type Na_2SO_4 (bivalent anion). When the salt in the capillary spaces of the gelatin granules was washed away with distilled water, the times of outflow were found to be about 97 seconds (varying between 95 and 102), regardless of the nature of the anion or cation (as long as the latter was univalent) of the salt used. This was an increase in the viscosity of about 20 per cent.

Second, if our hypothesis is correct, gelatin treated for 1 hour with M/8 or M/16 solutions of neutral salts of the alkaline earth metals should, when the salt solution in the capillary spaces of the powdered mass is washed away with H_2O, give times of outflow not much higher than those found for common gelatin (82 seconds). This also was found to be the case. Gelatin treated previously for 1 hour with M/8 or M/16 $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, gave outflows between 82 and 87, which is only a slight if any increase over that of common gelatin.

Gelatin treated with M/8 cane sugar, M/8 glycerol, or M/8 ethyl alcohol, gave times of outflow identical with those found for common gelatin. Table II gives the full results. The slight variations between members of the same group of salts are due to the fact that some viscosity determinations were made on the same day on which the gelatin was treated with the salt solution and others on the day following.

TABLE II.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution: gelatin treated with salt, then with H_2O to wash away salt.
			sec
I. Univalent cation, univalent anion.	M/8	NaCl	95
	M/8	$NaNO_3$	97
	M/8	Na acetate	95
	M/8	NaCNS	96
	M/8	KCl	99
	M/8	$LiNO_3$	96
	M/8	KCNS	96
	M/8	LiBr	99
	M/8	NH_4Br	96
II. Univalent cation, bivalent anion.	M/8	Na_2SO_4	95
	M/8	Na_2 oxalate	100
	M/16	Na_2 tartrate	99
	M/16	Na_2 malate	97
	M/16	Na_2 succinate	100
	M/16	Li_2SO_4	99
	M/16	K_2SO_4	99
	M/16	$(NH_4)_2SO_4$	98
	M/16	K_2 tartrate	102
III. Bivalent cation, univalent anion.	M/8	$MgCl_2$	87
	M/16	$CaCl_2$	82
	M/8	$SrCl_2$	87
	M/16	$BaCl_2$	82
IV. Bivalent cation, bivalent anion.	M/8	$MgSO_4$	90
V. Non-electrolytes.		H_2O	82
	M/8	Glycerol	79
	M/8	Cane sugar	80
	M/8	Ethyl alcohol	81

If it is true that the increase in viscosity is the expression of an increase in ionization of the gelatin, the facts found by us support our hypotheses: (1) that neutral salts with univalent cations form

with gelatin highly ionizable metal gelatinate compounds which dissociate into a positive metal and a negative gelatin ion; and (2) that neutral salts with bivalent cation form metal gelatinates which are considerably less ionizable. As far as the anion is concerned, its influence is neither noticeable in the additional swelling nor in the increase in viscosity. $MgSO_4$ and $CaSO_4$ act not differently from $MgCl_2$ and $CaCl_2$, and NaCl not differently from Na_2SO_4, as long as the concentration of the cation is the same. This means that the anion of the salt used either does not combine with the protein at all or that if it combines it enters into a bondage where its influence is not noticeable.

Finally, the results on viscosity support our statement drawn from the experiments on swelling that the action of neutral salts upon gelatin is of a stoichiometrical character, since the increase in the viscosity of the gelatin caused by a previous treatment with a salt solution is exactly the same for all salts with univalent cations regardless of the nature of the anion or cation as long as the concentration of the cation is the same. This stoichiometrical result is entirely independent of the hypothesis that the increase in viscosity is due to an increase in ionization.

IV.

If we treat gelatin with HCl, gelatin chloride (or hydrochloride) is formed, which according to our previous observations should react only with the anion of neutral salts, since such a compound dissociates preeminently or exclusively into a positive gelatin ion and a negative chlorion. Hence we should expect that a treatment of gelatin chloride with salts of the calcium group would affect the viscosity of gelatin chloride (after the salt solution is washed away) qualitatively and quantitatively like the treatment with salts of the type NaCl, provided the concentration of the Cl ion is the same; while the influence of the cations, Na and Ca, which was so strong in the case of common gelatin, should not be noticeable. All this turned out as expected. Hence neutral salts of the calcium group as well as neutral salts of the sodium group form highly ionizable salts with gelatin chloride—as long as the anion of the salt is univalent. Neutral salts with bivalent anion, however, should form with gelatin chloride new compounds

(gelatin sulfate, gelatin oxalate, gelatin malate, etc.) which are less ionizable than gelatin chloride (or hydrochloride), and this was also borne out by the experiments which we will now describe in more detail.

1 gm. of powdered gelatin was put for half an hour into a beaker containing 100 cc. M/100 HCl and the gelatin was repeatedly stirred. The gelatin was then put into the cylindrical funnel and the HCl solution (or what was left of it) allowed to filter off. After this the gelatin in the funnel was perfused three times with 25 cc. of the salt solution (M/8 or M/16 respectively) and then four times with 25 cc. of H_2O to remove any free salt solution. After this water had run off the gelatin was made up into a 1 per cent solution (in distilled water) and its viscosity was determined immediately as described.

The time of outflow of liquid gelatin chloride produced by treatment with M/100 HCl, when perfused three or four times with water to remove the remnant of acid in the capillary spaces between the gelatin granules, was for a 1 per cent solution generally between 120 and 130 seconds, and sometimes higher.

Gelatin chloride produced by a half hour treatment of 1 gm. of powdered gelatin in 100 cc. M/100 HCl was put into a funnel, the HCl solution was allowed to filter off, and the gelatin was perfused three times with M/8 $MgCl_2$ or $CaCl_2$ or $SrCl_2$, and then perfused four times with H_2O. The time of outflow varied between 132 and 135 seconds (Table III), a value which was identical with the one obtained when salts with a monovalent cation and anion were used for perfusion, such as NaCl, $NaNO_3$, KCl, KCNS, LiBr, NH_4Br, etc., the time of outflow varying in this case between 129 and 136 seconds (Table III). Only Na acetate proved to be an exception as was the case in similar experiments on swelling. This suggests that in the reaction between gelatin chloride and neutral salts only the anion of the neutral salts reacts with the gelatin or that at least the effect of the cation is not noticeable. This is exactly the same conclusion to which our experiments on swelling had led us. On the other hand, the same theory demanded that when gelatin chloride reacts with salts with bivalent anion, like SO_4, oxalate, etc., gelatin sulfate, gelatin oxalate, etc., should be formed with a much lower degree of ionization and hence a much lower degree of viscosity. This was also

found to be true. When gelatin chloride obtained by interaction for half an hour between 100 cc. M/100 HCl and 1 gm. gelatin was perfused three times with one of these salts with bivalent anion and then freed from the salt by washing four times with

TABLE III.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution; gelatin first treated with M/100 HCl, then with salt, then with H_2O to wash away salt.
			sec.
I. Univalent cation, univalent anion.	M/8	NaCl	131
	M/8	$NaNO_3$	136
	M/8	Na acetate	107
	M/8	KCl	131
	M/8	$LiNO_3$	130
	M/8	KCNS	129
	M/8	LiBr	132
	M/8	NH_4Br	132
II. Univalent cation, bivalent anion.	M/8	Na_2SO_4	105
	M/8	Na_2 oxalate	94
	M/16	Na_2 tartrate	102
	M/16	Na_2 malate	109
	M/16	Na_2 succinate	106
	M/16	$(NH_4)_2SO_4$	106
	M/16	K_2 tartrate	97
III. Bivalent cation, univalent anion.	M/8	$MgCl_2$	132
	M/8	$CaCl_2$	133
	M/8	$SrCl_2$	135
IV. Bivalent cation, bivalent anion.	M/8	$MgSO_4$	103

H_2O, the viscosity was lowered more than 20 per cent, the time of outflow varying between 94 and 109 seconds (Table III). Hence sulfates, oxalates, malates, succinates, act upon gelatin chloride in the same way as do Ca or Mg or Ba upon common gelatin; namely, forming little or less dissociable gelatin salts. This

agrees with the conclusion drawn from the experiments on the effect of neutral salts upon the swelling of gelatin.

The fact that gelatin treated with M/100 HCl has a higher viscosity (and swells more) than gelatin treated with M/8 NaCl may be due to the difference in the mass of gelatin salt formed in the two cases.

V.

A third test for our theory should consist in the proof that the influence of neutral salts upon the viscosity of sodium gelatinate produced by the action of NaOH upon 1 gm. of gelatin runs parallel to the influence of neutral salts upon the viscosity of common gelatin. The test turned out as our theory demanded.

1 gm. of finely powdered gelatin was put for half an hour into a beaker containing 100 cc. M/100 NaOH. The gelatin was then put into a funnel in order to allow the NaOH to filter off, and after this the gelatin was perfused three times with 25 cc. of an M/8 or M/16 salt solution and four times with 25 cc. of H_2O to wash out the salt solution from the capillary spaces. The results were as follows.

When gelatin is treated with M/100 NaOH the viscosity measured in times of outflow is raised from 82 seconds to from 115 to 118 seconds. When it is washed with water a slightly lower value is obtained, due possibly to the washing away of some of the NaOH formed by hydrolytic dissociation of sodium gelatinate with subsequent diminution of sodium gelatinate. The value 115 to 118 seconds is higher than the value obtained by the interaction between M/8 NaCl and gelatin, which was about 98 seconds, indicating that more sodium gelatinate is formed by the interaction of 1 gm. of gelatin with 100 cc. M/100 NaOH than with 100 cc. M/8 NaCl.

The theory which we developed on the basis of the swelling experiments demands that if sodium gelatinate produced by the action of M/100 NaOH be treated with a neutral salt with univalent cation the viscosity should be the same no matter what the nature of the anion or whether the latter is monovalent or bivalent, provided that the concentration of the cation is the same in all cases and provided that the salt in the capillary spaces between the granules of gelatin is washed away before the viscosity is

determined. The experiments confirmed this expectation. For salts of the type NaCl as well as for salts of the type Na_2SO_4, the viscosity measured in time of outflow was the same; namely, in the neighborhood of 118 (Table IV). This again indicates

TABLE IV.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution; gelatin first treated with M/100 NaOH, then with salt, then with H_2O to wash away salt.
			sec.
I. Univalent cation, univalent anion.	M/8	NaCl	115
	M/8	$NaNO_3$	119
	M/8	Na acetate	120
	M/8	KCl	120
	M/8	$LiNO_3$	116
	M/8	KCNS	116
	M/8	LiBr	118
	M/8	NH_4Br	110
II. Univalent cation, bivalent anion.	M/16	Na_2SO_4	116
	M/16	Na_2 oxalate	112
	M/16	Na_2 tartrate	120
	M/16	Na_2 malate	120
	M/16	Na_2 succinate	119
	M/8	$(NH_4)_2SO_4$	110
	M/16	K_2 tartrate	117
III. Bivalent cation, univalent anion.	M/16	$MgCl_2$	87
	M/16	$CaCl_2$	96
	M/8	$SrCl_2$	98
IV. Bivalent cation, bivalent anion.	M/8	$MgSO_4$	103

that only the cation of the neutral salt interacts with sodium gelatinate. When $MgCl_2$, $CaCl_2$, and $SrCl_2$ were substituted for the salts with univalent cation the time of outflow was reduced to from 87 to 98 seconds (Table IV), indicating that lowering of the degree of electrolytic dissociation which our theory demanded.

This lowering was due to the formation of non- or less dissociable calcium gelatinate or magnesium gelatinate from sodium gelatinate.

VI.

Attention had been called in this and the writer's former papers to the fact that the additional swelling due to the action of a neutral salt upon gelatin is only noticeable after the excess of salt solution has been washed away, and this was explained on the assumption that the additional swelling was due to the gelatin ions formed under the influence of the salt, but owing to the repression of ionization in the presence of the salt the additional swelling could only show itself after the excess of salt solution was washed away. The same is true for the influence of salts upon the viscosity. When we measure the influence of neutral salts upon the viscosity of gelatin solution in the presence of the salt we get negative if not confusing results. Thus measurements of the viscosity of gelatin in M/8 $MgCl_2$, $CaCl_2$, or $SrCl_2$ gave times of outflow between 74 and 85 seconds, while the viscosity measurements of gelatin in the presence of salts of the type NaCl gave values between 72 and 76 seconds, and in the presence of salts of the type Na_2SO_4 the times were 80 to 83 seconds. Almost similar figures were obtained for gelatin treated first with M/100 HCl and then with M/8 neutral salts. This agrees with our theory that the effect of neutral salts upon the additional swelling and upon the increase of viscosity is due to the ionization of gelatin salts with univalent anion or cation; and that this ionization is repressed by the presence of as high a concentration of a neutral salt as M/8 or M/16. This confirms our statement that any theory of the action of the neutral salts upon proteins must be based on experiments in which the excess of salts which have acted on the protein has been washed away.

VII.

We have called attention to the fact that if dried pig's bladder or powdered gelatin is treated with 100 cc. M/8 NaCl containing quantities of M/8 $CaCl_2$ varying from 1 to 32 cc. the additional swelling noticeable after the salt is washed away diminishes with

the amount of $CaCl_2$ added. This is a case of antagonistic salt action. It was of interest to find out whether this antagonistic effect of the addition of $CaCl_2$ to NaCl would also become noticeable in the viscosity of the gelatin after the salt was washed away. This was the case. 1 gm. of powdered gelatin was put for 1 hour into 100 cc. M/8 NaCl containing 0, 1, 2, 4, 8, 12, 16, and 32 cc. M/8 $CaCl_2$. As controls gelatin treated with H_2O alone and gelatin treated for 1 hour with M/8 $CaCl_2$ were added. After staying 1 hour in these solutions the gelatin was put into the cylindrical funnels where it was washed four times with 25 cc. of distilled water, until the filtrate no longer gave a precipitate with silver nitrate. The additional swelling (expressed in mm. of the height of the cylindrical column of gelatin), the viscosity expressed in time of outflow, and the alcohol precipitability of 1 per cent gelatin solutions treated in the way mentioned are given in Table V. The reader's attention is called to the fact that all the measurements were made on gelatin washed free from the salt originally used and dissolved in H_2O.

TABLE V.

1 gm. gelatin put for 1 hour into the following solutions and then put into funnels and perfused four times with distilled water.

	100 cc. M/8 NaCl + cc. M/8 $CaCl_2$.							M/8 $CaCl_2$	H_2O
	0	1	2	4	8	16	32		
Additional swelling in mm. of the cylindrical column of gelatin...	36	38	35	25	23	22	17	6.0	6.5
Viscosity of 1 per cent gelatin solution in H_2O in seconds of time of outflow.....	98	96	96	92	91	88	90	88	84
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H_2O.	∞	∞	∞	∞	About 20	9.5	6.5	5.4	5.5

The additional swelling is markedly lowered by the addition of 4 cc. or more of M/8 $CaCl_2$ to 100 cc. M/8 NaCl, and the addition of 1 cc. or more of $CaCl_2$ lowers the viscosity. The precipitability with alcohol begins when more than 8 cc. M/8 $CaCl_2$ is added to 100 cc. M/8 NaCl.

Viscosity and additional swelling give parallel results, supporting our view that in this case the antagonism is due to the diminution in the ionization of a metal gelatin through the transformation of the highly ionizable sodium gelatinate into calcium gelatinate which is only slightly ionizable.

There exists a second type of antagonistic salt action due to the transformation of the highly ionizable gelatin chloride into the less ionizable gelatin sulfate. It was of interest to apply the three tests, additional swelling, viscosity measurements, and precipitation with alcohol, to this assumption.

1 gm. of powdered gelatin was put for half an hour into each of a series of beakers containing 100 cc. M/100 HCl to transform the gelatin into gelatin chloride or hydrochloride. The gelatin was then put into a series of cylindrical funnels which were perfused three times with 25 cc. of an M/8 solution of NaCl containing varying quantities of M/16 Na_2SO_4; namely, the following mixtures, 100 cc. M/8 NaCl containing 0, 1, 2, 4, 8, 16, and 32 cc. M/16 Na_2SO_4. As a control one funnel was perfused with M/16 Na_2SO_4. After this each funnel was perfused four times with 25 cc. H_2O to wash away the salt solution in the capillary spaces between the granules.

Table VI gives the results. The additional swelling is the swelling observed at the end of the washing with water, in excess of the swelling which occurred in the gelatin while the latter was in the acid solution. The reader will notice that the addition of Na_2SO_4 to NaCl diminishes the ionizing effect of the NaCl, inasmuch as with the increase in the quantity of Na_2SO_4 added to the NaCl solution the additional swelling, as well as the viscosity, is diminished (Table VI).

It is obvious again that the values for viscosity are extremely regular and that they diminish with the amount of Na_2SO_4 added. The viscosity of a 1 per cent gelatin solution only perfused with H_2O corresponded to a time of outflow of 86 seconds.

These viscosity measurements therefore support our hypothesis that antagonistic salt action can be produced by the transformation of a more ionizable gelatin salt of the type sodium gelatinate into a less ionizable gelatin salt of the type calcium gelatinate; or of the more ionizable salt of the type gelatin chloride into a less ionizable salt of the type gelatin sulfate.

TABLE VI.

1 gm. gelatin put for 30 min. into 100 cc. M/100 HCl to produce gelatin chloride and then put into funnels and perfused three times with 25 cc. of the following salt solutions and four times with 25 cc. H_2O to wash away the salt.

	100 cc. M/8 NaCl + cc. M/16 Na_2SO_4.							M/16 Na_2SO_4
	0	1	2	4	8	16	32	
Additional swelling in mm. of the height of the cylindrical column of gelatin	36	34	29	25.5	26.5	24	14	−5.5
Viscosity of 1 per cent gelatin solution in H_2O in seconds of time of outflow	138	137	135	131	130	125	118	108
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H_2O	∞	∞	∞	∞	∞	∞	∞	9

Theoretical Remarks.

In three former papers we have shown that when powdered gelatin or dried pig's bladder is treated with the solution of a neutral salt, the gelatin or bladder undergoes a certain amount of swelling and that after the salt solution is washed away with distilled water a considerable additional swelling of the gelatin occurs. In the same papers we had also shown that the influence of neutral salts upon the additional swelling of gelatin or pig's bladder is of a stoichiometrical character, depending purely upon the concentration and valency of the cation of the salt used. Our present experiments on the influence of neutral salts upon the viscosity of gelatin solution show that the influence of neutral salts upon the viscosity is also of a stoichiometrical character, inasmuch as it also depends exclusively or preeminently upon the concentration and valency of the cation of the neutral salt used. The anion seemed to be without influence. The writer had found in his former experiments that the neutral salts with monovalent cation cause a considerable additional swelling of the gelatin while the neutral salts with bivalent cation cause no such swelling, and to explain this difference he had assumed that neutral salts with monovalent cation form with gelatin metal

gelatinates which are highly ionizable, while salts with bivalent cations form with gelatin metal gelatinates which are much less or only slightly ionizable. The experiments on viscosity support this conclusion, if the statement generally made is correct that ionization increases the viscosity of a protein solution.

In publications on the peculiarities of colloids the statement is frequently made that the oppositely charged ions of a neutral salt affect a colloid, *e.g.* a protein, in the opposite sense and that the effect of the salt is always the algebraic sum of these two opposing forces. This statement does not hold for the influence of neutral salt on those qualities of colloids which have thus far formed the subject of our investigation; namely, additional swelling, viscosity, and precipitability by alcohol after the salt has been washed away. Common gelatin (and the same may be true for all proteins which are much stronger as acids than as bases) reacts with neutral salts as if only the cation of the salt were active; salts of the type NaCl and of the type Na_2SO_4 act qualitatively and quantitatively alike as long as the concentration of the cation is the same, and the nature of the anion does not influence the result. It was also possible to show that $MgCl_2$ acts like $MgSO_4$ and $CaCl_2$ like $CaSO_4$ as long as the concentration of the cation is the same. All this would be impossible if the anion acted upon the protein in a sense opposite to that of the cation. The qualities which we have investigated depend apparently on the ionized negative gelatin ion and this combines only with the cation of the neutral salt applied. If the anion of the latter enters at all into combination with the negative gelatin ion its influence does not show itself in those qualities which thus far have formed the subject of our investigations.

When we produce gelatin chloride (or hydrochloride) by treating the gelatin with M/100 HCl, and wash away the superfluous acid the gelatin chloride is only influenced by the anion of a neutral salt, and salts of the type $CaCl_2$ act qualitatively and quantitatively like salts of the type NaCl if the concentration of the Cl ion is the same. The cation does not act in the opposite sense from the anion of the salt but does not act at all, or at least its influence is not noticeable, and Ca, which influences common gelatin and sodium gelatinate produced by treatment with NaOH so powerfully, is apparently without influence when acting upon

gelatin chloride. Our experiments have therefore led to a remarkable simplification of our conception of the influence of neutral salts upon proteins inasmuch as they show that for the physical characters thus far studied by us only one of the ions of the neutral salts is to be considered; namely, the one which has the opposite charge from the protein ion with which it reacts.

There are other qualities of proteins which are not confined to the ionized protein. Our investigations have not yet been extended to these qualities and we are not prepared to say whether or not qualities depending upon the non-dissociated protein molecules are influenced in an opposite sense by the oppositely charged ions of neutral salts.

SUMMARY.

1. Our experiments have shown that when we treat gelatin with M/8 or M/16 solutions of neutral salts with univalent cation, the viscosity of the gelatin is increased considerably after the excess of salt is washed away. When we treat gelatin with M/8 or M/16 solutions of neutral salts with bivalent cation no or only a slight increase of viscosity of the gelatin is noticeable after the excess of salt is washed away.

2. Neutral salts with univalent cation (Li, Na, K, NH_4) produce the same increase in the viscosity of the gelatin solution as long as the concentration of cation is the same,—namely M/8 in our experiments,—regardless of the nature of the anion and regardless of whether the anion is univalent or bivalent.

3. The statements made concerning the influence of neutral salts upon the viscosity of gelatin hold also for the influence of these salts upon the viscosity of gelatin previously treated with an alkali; *e.g.*, NaOH.

4. The action of neutral salts upon the viscosity of gelatin previously treated with HCl shows that salts with bivalent anion lower the viscosity of such gelatin; while salts with univalent anion have the opposite effect. The influence of the cation was not noticeable in our experiments.

5. These observations do not agree with the statement frequently made that the action of a neutral salt upon a protein is the algebraic sum of the opposite action of the oppositely charged

ions of the neutral salts. The viscosity of a gelatin solution is influenced chiefly if not exclusively by one of the two ions of the neutral salt; namely, the one which has the opposite electrical charge from that of the protein. Since common gelatin is stronger as acid than as base, its viscosity is influenced only by the cation of a neutral salt; and the same is true for the influence of neutral salts upon the viscosity of metal gelatinates produced by the action of bases upon gelatin. The viscosity of gelatin-acid salts (produced by the action of acids upon gelatin) which yield a positive gelatin ion is influenced chiefly if not exclusively by the anion of a neutral salt.

6. These conclusions are in agreement with the conclusions drawn from the experiments on the limiting concentration of neutral salts upon additional swelling reported in the writer's previous publications.

7. If it is true that the increase in the viscosity of gelatin solution under the influence of electrolytes is due to an increase in the ionization of the gelatin, our results support the hypothesis that neutral salts with univalent cation form highly ionizable metal proteinates with proteins of the type of gelatin (which is much stronger as acid than as base), while neutral salts with bivalent cation form less or non-dissociable metal proteinates with gelatin.

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

IV. THE PRECIPITATION OF GELATIN BY MIXTURES OF SALTS.

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The precipitation of colloids by electrolytes has probably been studied more extensively than any other single problem in this field. There are still many obscurities to be cleared up, as for example why increase of temperature sometimes increases and sometimes decreases precipitation. A few general conclusions, however, have been established beyond question.

The chief requirement for precipitation seems to be that the colloid shall be electrically neutral. In the case of the hydrophil colloids which are closely associated with water, a second factor becomes equally important; *i.e.*, a sufficient salt concentration to "salt out" or dehydrate the protein. Chick and Martin[1] have distinguished between these two factors in studying the precipitation of egg albumin by electrolytes. They found that when the dehydrating capacity was supplied by alcohol, the albumin was precipitated by small concentrations of salts and in accordance with Hardy's[2] valence rule. Only small amounts of the precipitating agents were required, as in the precipitation of the suspension colloids. Hardy also established the fact that precipitation is accomplished by the ion of opposite charge to that on the colloid particle. This has been confirmed by all subsequent workers, particularly by Pauli.[3]

[1] Chick, H., and Martin, C. J., *Biochem. J.*, 1913, vii, 380.

[2] Hardy, W. B., *Z. physikal. Chem.*, 1900, xxxiii, 385.

[3] Pauli, W., and Rona, P., *Beitr. chem. Physiol. u. Path.*, 1902, ii, 1. Pauli, W., *ibid.*, 1903, iii, 225; 1905, vi, 233; 1906, vii, 531; *Arch. ges. Physiol.*, 1899-1900, lxxviii, 342.

The precipitation of colloids in mixtures of electrolytes has been confined chiefly to the precipitation by salts in acid, alkaline, and neutral solutions. Linder and Picton,[4] however, investigated the precipitation of arsenious sulfide sols in mixtures of salts. Blake[5] did some experiments of this sort on colloidal gold, Bender[6] and Bechhold[7] on mastic suspensions, and Pauli[8] on egg albumin. Koch and Williams[9] have investigated the effects of salts on the precipitation of lecithin by $CaCl_2$.

The present paper comprises a description of some experiments by the writer on the precipitation of gelatin by mixtures of electrolytes, as a sequel to those already published[10,11,12] on the precipitation of gelatin by alcohol from solutions of electrolytes and mixtures of electrolytes.

The method adopted for this investigation was to add NaCl in solid form to test-tubes containing 5 cc. of 2 per cent gelatin (dissolved in solutions of electrolytes of varying concentrations) until the point of precipitation was reached. During the experiment the tubes were kept at constant temperature in a water bath at 30 ± 1°C., as an increase in temperature was found to assist the precipitation by NaCl very markedly. After the point of precipitation was reached, the contents of the tubes were diluted to 100 cc. in a volumetric flask. This diluted the gelatin so much that it was possible to titrate the solution for chlorides with standard $AgNO_3$ and K_2CrO_4 without appreciable error. In presence of stronger gelatin solutions this method always gives a higher chloride content than actually exists, due to the fact that the formation of the red Ag_2CrO_4 at the end-point is retarded, probably because of combination with the gelatin. If the temperature is kept constant to within 1° and ordinary care is used in the

[4] Linder, S. E., and Picton, H., *J. Chem. Soc.*, 1895, lxvii, 63.

[5] Blake, J. C., *Am. J. Sc.*, 1903, xvi, 433.

[6] Bender, R., *Kolloid-Z.*, 1914, xiv, 255.

[7] Bechhold, H., *Z. physikal. Chem.*, 1904, xlviii, 385.

[8] Pauli, *Beitr. chem. Physiol. u. Path.*, 1904, v, 27.

[9] Koch, W., and Williams, A. W., *J. Pharmacol. and Exp. Therap.*, 1910-11, ii, 253.

[10] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279.

[11] Fenn, *J. Biol. Chem.*, 1918, xxxiii, 439.

[12] Fenn, *J. Biol. Chem.*, 1918, xxxiv, 141.

addition of NaCl to the gelatin, this method gives results which may be duplicated to within 2 per cent.

The empirical conclusion drawn from these experiments is that NaCl behaves like a salt in which the effect of the anion predominates over that of the cation. *Precipitation is therefore most*

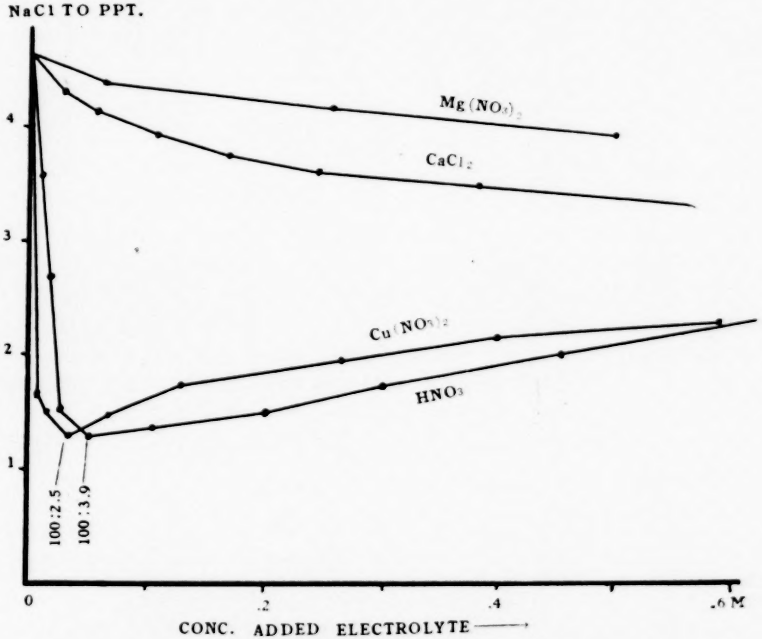

FIG. 1. Abscissæ represent concentrations of electrolytes added to the gelatin. The ordinates show the molecular concentration of NaCl necessary to precipitate the gelatin. The curves show that salts with prepotent cations assist precipitation by NaCl. The molecular proportions of Na : Cu and Na : H at the minima are 100 : 2.5 and 100 : 3.9 respectively.

assisted by those electrolytes which make the gelatin most electro-positive and most hindered by those which make it most electro-negative.

The effect of salts with predominant cations on the precipitation produced by NaCl is shown in Fig. 1. On the abscissæ are plotted molecular concentrations of the electrolytes in the gelatin, and on the ordinates the molecular concentrations of

NaCl which were necessary to precipitate. Under the conditions of the experiment, pure gelatin was precipitated by NaCl at a concentration of 4.65 to 4.75 M. In the presence of small amounts of HNO_3 or $Cu(NO_3)_2$, NaCl 1.3 M is sufficient. More than this

TABLE I.

Effect of Various Cations on the Precipitation of Gelatin by NaCl.

$Cu(NO_3)_2$		HNO_3		$CaCl_2$		$Ca(NO_3)_2$		$Mg(NO_3)_2$	
Concentration of $Cu(NO_3)_2$.	NaCl to precipitate.	Concentration of HNO_3.	NaCl to precipitate.	Concentration of $CaCl_2$.	NaCl to precipitate.	Concentration of $Ca(NO_3)_2$.	NaCl to precipitate.	Concentration of $Mg(NO_3)_2$.	NaCl to precipitate.
M	M	M	M	M	M	M	M	M	M
2.0	2.83	1.07	3.12	0.86	3.19	0.44	3.36	1.0	3.77
1.33	2.56	0.802	2.51	0.575	3.36	0.32	3.52	0.5	3.96
0.89	2.52	0.603	2.30	0.383	3.47	0.248	3.63	0.25	4.17
0.591	2.30	0.45	2.02	0.245	3.60	0.185	3.82	0.0625	4.38
0.394	2.18	0.301	1.75	0.17	3.73	0.139	3.87	0.0	4.65
0.262	1.96	0.201	1.51	0.113	3.93	0.104	4.06		
0.131	1.74	0.1	1.38	0.0566	4.15	0.052	4.27		
0.0655	1.44	0.05	1.28	0.0283	4.32	0.026	4.52		
0.0327	1.30	0.025	1.56	0.0	4.65	0.0	4.77		
0.0163	1.49	0.0125	2.73	0.0	4.73				
0.0081	1.85	0.00625	3.62						
0.0	4.65	0.0	4.64						

5 cc. of a 2 per cent solution of gelatin dissolved in an acid or salt in the concentration noted in the first columns are kept in a test-tube in a water bath at 30 ± 1°C. and dry NaCl is added until the gelatin is precipitated. The concentration of the NaCl in the solution is then determined by diluting in a 100 cc. volumetric flask and titrating samples with $AgNO_3$ and K_2CrO_4 for chlorides. The concentration of NaCl so determined is noted in the second columns. The purest commercial gelatin obtainable was used, designated in previous papers as gelatin A. The precipitate with NaCl is always increased by heating except in 0.5 M $Cu(NO_3)_2$ and 0.05 M HNO_3 or higher concentrations of these electrolytes.

amount of $Cu(NO_3)_2$ or HNO_3 tends to hinder precipitation again. This point of optimum precipitation is probably an isoelectric point. The molecular proportions of Na : Cu and Na : H are 100 : 2.5 and 100 : 3.9, respectively, as inserted on the figure.

A number of similar combinations of electrolytes have been tried by adding the NaCl or $NaNO_3$ to the gelatin in concentrated solutions instead of in solid form. Figures for these experiments are given in Table IV and a typical curve is plotted in Fig. 4 for NaCl and HCl. In all these cases a more or less distinct isoelectric point is found. At this point the molecular proportions are 100 : 1.1 for NaCl plus HCl; 100 : 0.4 for NaCl plus $AlCl_3$; 100 : 0.77 for $NaNO_3$ plus $AlCl_3$; and 100 : 1.1 for NaCl plus $La_2(NO_3)_6$. These proportions can be determined only roughly by this method but they suggest a certain balance between the positive and the negative ions.

Whether or not this is the correct explanation, it is well known from the literature that NaCl precipitates proteins more easily in acid solution and that with a constant salt content there is an optimum acid concentration for precipitation. Examples of this for globulins are given by Hardy[13] and by Mellanby,[14] and for egg albumin by Chick and Martin.[15] Mention may also be made of similar experiments by Mörner (1899)[16] on the precipitation of gelatin by mixtures of acetic acid and $K_4Fe(CN)_6$. Similar effects have been described by Mines[17] for the precipitation of blood corpuscles, and by the writer[18] for the precipitation of milk and olive oil emulsions. Wood and Hardy[19] have shown that there is an optimum acidity for the stability of doughs containing a constant amount of NaCl. Hardy[13] has suggested that this striking difference between the effects of acids and alkalies on NaCl in protein solutions is due to the fact that the acids and salts unite with the protein in the same way but that the alkalies and salts unite in different ways.

In Fig. 1 are also shown curves for the effect of $CaCl_2$ and $Mg(NO_3)_2$ on the precipitation by NaCl. Both of these salts assist precipitation by the effect of their cations. The $CaCl_2$ curve was carried out to a concentration of 0.86 M and the $Mg(NO_3)_2$

[13] Hardy, *J. Physiol.*, 1905–06, xxxiii, 251.
[14] Mellanby, J., *J. Physiol.*, 1905–06, xxxiii, 338.
[15] Chick and Martin, *Kolloidchem. Beihefte*, 1913–14, v, 50.
[16] Mörner, C. T., *Z. physiol. Chem.*, 1899, xxviii, 471.
[17] Mines, G. R., *Kolloidchem. Beihefte*, 1911–12, iii, 191.
[18] Fenn, *Proc. Nat. Acad. Sc.*, 1916, ii, 534.
[19] Wood, T. B., and Hardy, W. B., *Kolloid-Z.*, 1909, iv, 213.

curve to a concentration of 1 M but no minimum like that found for $Cu(NO_3)_2$ was observed. In the $CaCl_2$ curve, allowance was made (in determining the amount of NaCl added) for the chlorides of Ca already present. $Ca(NO_3)_2$ gave similar results.

The effect of various anions is shown in Fig. 2. Salts like Na_3 citrate and Na acetate, which are alkaline in solution, cause

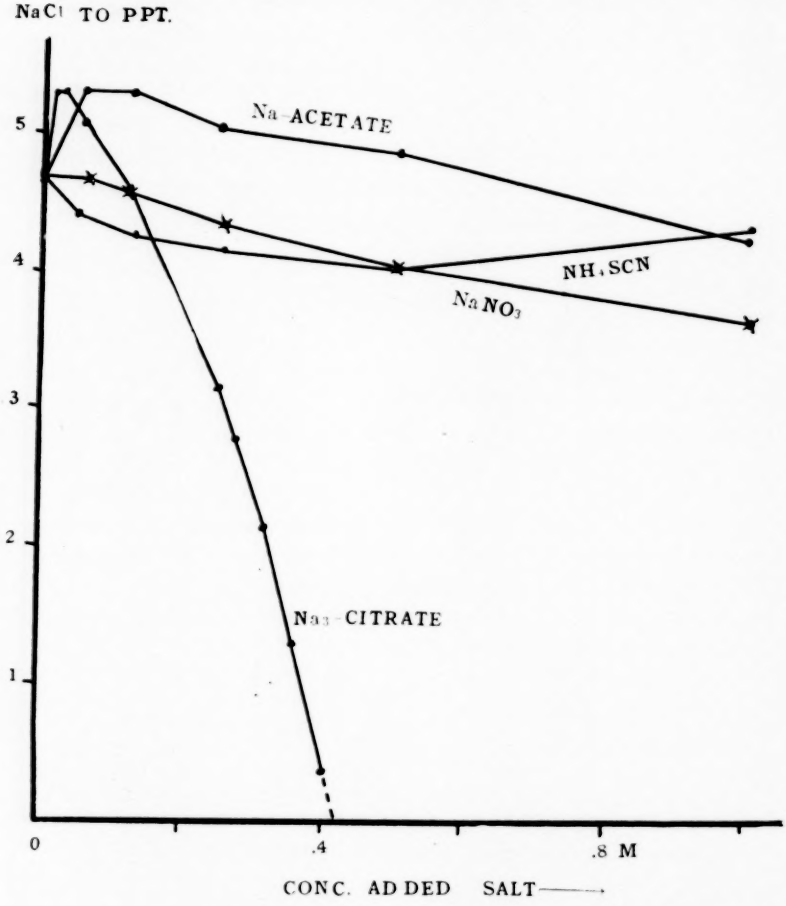

FIG. 2. Curves showing the effect of various anions on the precipitation of gelatin by NaCl. Ordinates and abscissæ as in Fig. 1. Salts which charge the gelatin negatively tend to hinder precipitation.

TABLE II.

Effect of Various Anions on the Precipitation of Gelatin by NaCl.

$NaNO_3$		KSCN		NH_4SCN		Na acetate.		Na_3PO_4		Na_2SO_3		Na citrate.			NH_4 tartrate.	
Concentration of $NaNO_3$.	NaCl to precipitate.	Concentration of KSCN.	NaCl to precipitate.	Concentration of NH_4SCN	NaCl to precipitate.	Concentration of Na acetate.	NaCl to precipitate.	Concentration of Na_3PO_4.	NaCl to precipitate.	Concentration of Na_2SO_3.	NaCl to precipitate.	Concentration of Na citrate.	NaCl to precipitate.		Concentration of NH_4 tartrate.	NaCl to precipitate.
													(a)	(b)		
M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
2.0	2.94	1.5	4.24	2.0	5.25	2.0	2.84	0.5	0.0	0.8	0.0	0.41	0.383	0.39	0.641	0.0
1.0	3.65	0.75	3.77	1.0	4.28	1.0	4.25	0.25	3.47	0.4	3.22	0.354	1.35	1.35	0.55	0.225
0.5	4.01	0.375	3.86	0.5	4.01	0.5	4.86	0.125	5.05	0.2	4.64	0.313	2.19	2.14	0.514	0.526
0.25	4.30	0.188	3.97	0.25	4.18	0.25	5.03	0.0625	5.11	0.1	5.11	0.278	2.75	2.67	0.481	0.71
0.125	4.55	0.094	4.34	0.125	4.25	0.125	5.27	0.0312	5.49	0.05	5.45	0.25	3.15	—	0.428	1.07
0.0625	4.66	0.047	4.39	0.0625	4.40	0.0625	5.33	0.0156	5.35	0.025	5.42	0.125	4.58	4.83	0.385	1.44
												0.0625	5.04	5.03	0.257	2.92
												0.0312	5.31	5.10	0.149	—
												0.0156	5.30	—	0.074	4.52
												0.0078	5.05	5.04	0.037	4.62
												0.0	4.75	4.55		

Tabulation and method of experimentation is the same as in Table I. See Fig. 2.

first an increase in the amount of NaCl required to precipitate, and in higher concentrations a decrease. Curves similar to the Na_3 citrate curve are also obtained with Na_3PO_4, Na_2SO_3, and NH_4 tartrate. Figures are given in Table II. If all the curves were prolonged until they crossed the base line, *i.e.* until they precipitated the gelatin without NaCl, the salts would arrange themselves in the lyotropic series. As $NaNO_3$ precipitates pure gelatin at a concentration of about 6 M and Na acetate at about 3 M, it is evident that the Na acetate curve must cross both the $NaNO_3$ and the NaCl curves at some point not shown in the figure. (The NaCl curve is not plotted but it would be a straight line crossing the base line at a concentration of 4.65 M since pure gelatin is precipitated by NaCl 4.65 M.) These crossings, together with those shown in the figure for the NH_4SCN curve (which never reaches the base line) should warn the investigator not to expect uniformity in the lyotropic series.

In NaOH no precipitate can be obtained with NaCl except at very low concentrations. If, however, 1 cc. of 18 per cent gelatin is added to 5 cc. of a solution of NaOH 1 M or stronger (particularly 5 M) a white coagulum is obtained which disappears gradually as the gelatin is hydrolyzed by the strong alkali. Similarly gliadin of wheat flour is precipitated by high concentrations of both NaOH and HCl but this precipitate disappears on standing a few hours. No such precipitation of gelatin by strong HCl has been observed.

In Fig. 3 is shown the effect of various sulfates on the precipitation by NaCl. These curves bear out the general conclusions of this paper in that the sulfates of the bivalent cations, as Zn, Mg, Fe, and Mn, assist the precipitation by NaCl, these curves being convex to the base line, while the sulfates of monovalent cations, as Na_2SO_4, hinder precipitation (this curve being concave to the base line). A little consideration will show the validity of this criterion of the favorable or unfavorable effect of a salt on the precipitation by NaCl, since the curve for a salt which neither assisted nor hindered precipitation by NaCl would be a straight line connecting the points on the two axes where each of the two salts would cause precipitation in the absence of the other. Such a straight line is drawn (dotted) between the ends of the Na_2SO_4 curve to emphasize the fact that the effect of the SO_4 ion alone is to prevent the precipitation by NaCl.

In a previous paper[11] the writer showed that the presence of NaCl in gelatin would decrease the effect of both $CaCl_2$ and Na_2SO_4 in hindering the precipitation of gelatin by alcohol, but that $CaCl_2$ and Na_2SO_4 differed in that small concentrations of the former could decrease the effect of NaCl but that small concentrations of the latter could not. In the present paper,

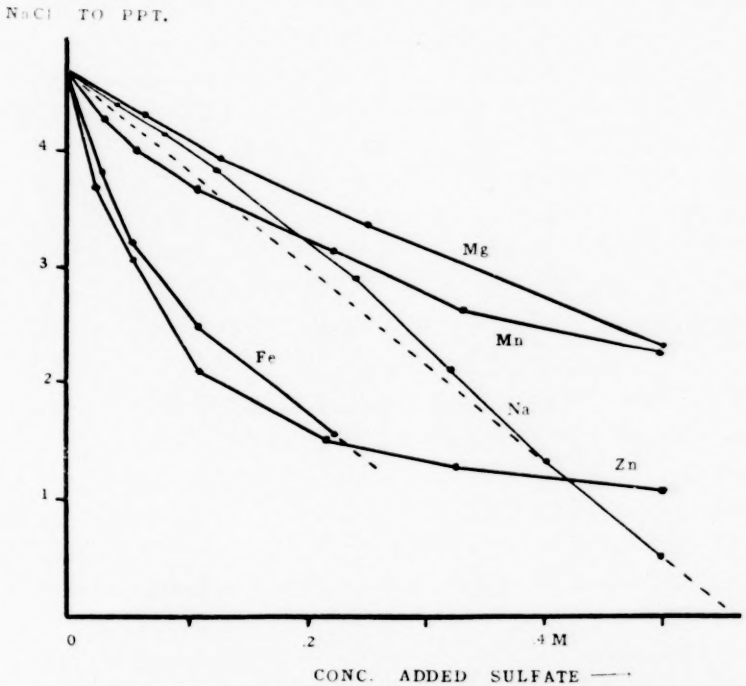

FIG. 3. Curves showing the effect of various sulfates on the precipitation of gelatin by NaCl. Ordinates and abscissæ as in Fig. 1. Na_2SO_4 tends to hinder precipitation by NaCl but sulfates with bivalent cations assist it.

it is shown that $CaCl_2$ assists while Na_2SO_4 hinders the precipitation of gelatin by NaCl. Both of these results are in agreement in that NaCl acts in both cases as if the Cl ion were more strongly combined with the gelatin than the Na ion. In some measure supporting this conclusion, may be mentioned the fact that the isoelectric point of gelatin was determined by

Chiari[20] as 2×10^{-5} and by Michaelis and Grineff[21] as $2.3\times10,^{-3}$ indicating a stronger dissociation of H ions than of OH ions. The excess of H ions resulting would hinder the Na ion from combining as readily as the Cl ion. Hopkins and Savory,[22] furthermore, found that the Bence-Jones protein from pathological urine could be precipitated more easily from acid solution than from alkaline

TABLE III.

Effect of Various Sulfates on the Precipitation of Gelatin by NaCl.

Na_2SO_4			$MgSO_4$		$ZnSO_4$		$FeSO_4$		$MnSO_4$	
Concentration of Na_2SO_4.	NaCl to precipitate.		Concentration of $MgSO_4$.	NaCl to precipitate.	Concentration of $ZnSO_4$.	NaCl to precipitate.	Concentration of $FeSO_4$.	NaCl to precipitate.	Concentration of $MnSO_4$.	NaCl to precipitate.
	(a)	(b)								
M	M	M	M	M	M	M	M	M	M	M
0.5	0.51	—	1.0	0.238	0.5	1.08	0.33	0.0	0.5	2.25
0.4	1.39	1.29	0.5	2.28	0.33	1.26	0.22	1.57	0.33	2.60
0.32	2.12	2.05	0.25	3.38	0.22	1.52	0.11	2.52	0.22	3.11
0.24	2.86	2.88	0.125	3.90	0.11	2.13	0.055	3.21	0.11	3.63
0.18	3.43	3.33	0.0625	4.29	0.055	3.08	0.027	3.86	0.055	4.01
0.12	3.80	3.89	0.0	4.61	0.027	3.67			0.027	4.28
0.08	4.14	4.16								
0.04	4.38	4.42								
0.02	4.59	4.56								
0.01	4.60	4.61								
0.0	4.69	4.74								
0.0	4.70	4.71								

Two determinations were made for each concentration of Na_2SO_4 and their average was plotted in Fig. 3.

The method of tabulation and experimentation is the same as in Table I.

solution and suggested that the protein takes a negative charge more readily than a positive one. They find also that NaCl acts more like Na_2SO_4 than like $CaCl_2$.

The effect of acids and alkalies on the precipitation of gelatin

[20] Chiari, R., *Biochem. Z.*, 1911, xxxiii, 167.

[21] Michaelis, L., and Grineff, W., *Biochem. Z.*, 1912, xli, 373.

[22] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

by Na_2SO_4 is shown in Fig. 5. In these experiments 5 cc. of a 3 per cent solution of gelatin in HCl or NaOH were titrated with a molecular solution of Na_2SO_4 until precipitation occurred. HCl assists the precipitation, as expected, the turning point of the curve being probably an isoelectric point, where the proportions of SO_4 to H are 100 : 2. A similar experiment with $AlCl_3$ yields

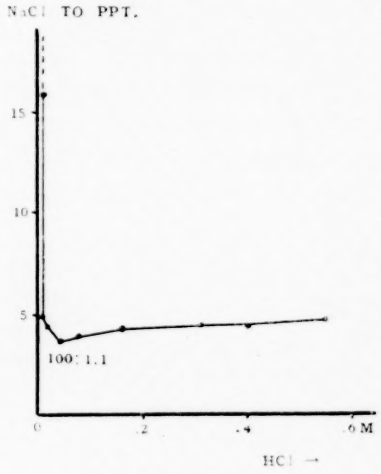

FIG. 4.

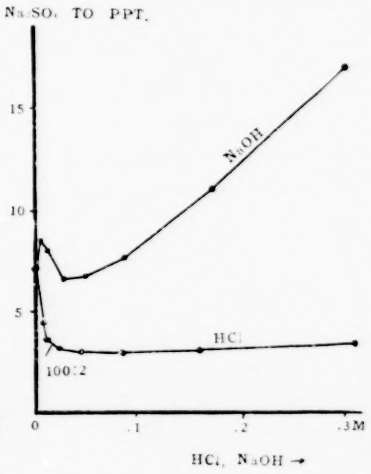

FIG. 5.

FIG. 4. Curves showing the effect of increasing concentrations of HCl in gelatin on the amount of NaCl 4.24 M which is necessary to precipitate it. The molecular proportions of Na : H at the minimum (probably an isoelectric point) are 100 : 1.1.

FIG. 5. Curves showing the effect of NaOH and HCl on the precipitation of gelatin by Na_2SO_4. Abscissæ show the concentrations of HCl or NaOH in the gelatin before addition of Na_2SO_4.

essentially the same result, the proportions at the turning point of the curve being about 100: 1. Data for this experiment are given in Table V.

The NaOH curve in Fig. 5 shows that small amounts of NaOH hinder the precipitation of gelatin by Na_2SO_4, larger amounts assist it, and still higher concentrations hinder it (due to the decomposition of the gelatin by the alkali). Figures for a similar curve, using Na_3 citrate instead of Na_2SO_4, are given in Table V

and this result has also been confirmed by dissolving gelatin in a representative series of mixtures of NaOH and Na_2SO_4 and

TABLE IV.

Effect of Acid and Trivalent Cations on the Precipitation of Gelatin by Monovalent Sodium Salts.

Precipitation by $NaNO_3$ of gelatin plus $AlCl_3$.		Precipitation by NaCl of gelatin plus $AlCl_3$.		Precipitation by NaCl of gelatin plus HCl.		Precipitation by NaCl of gelatin plus $La_2(NO_3)_6$.	
Concentration of $AlCl_3$ in gelatin.	$NaNO_3$ 6 M to precipitate.	Concentration of $AlCl_3$ in gelatin.	NaCl 4 M to precipitate.	Concentration of HCl in gelatin.	NaCl 4.24 M to precipitate.	Concentration of $La_2(NO_3)_6$ in gelatin.	NaCl 3.98 M to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.
1.5	5.0	0.833	17.7	6.2	17.5	0.35	6.8
0.75	6.2	0.63	17.8	3.8	13.8	0.17	7.0
0.375	6.0	0.37	15.7	2.8	12.1	0.083	6.1
0.188	5.5	0.24	14.7	2.1	10.1 8.5	0.041	5.4
0.094	4.5	0.13	13.6	1.67	7.2	0.02	6.1
0.047	4.0	0.092	12.2	1.6	8.9	0.011	9.6
0.023	3.0	0.042	10.2	1.25	6.15	0.0055	No ppt.
0.012	3.2	0.021	8.1	1.00	5.7	0.0	" "
0.006	4.0	0.01	10.4	0.63	5.2		
0.003	No ppt.	0.005	17.7	0.52	4.7		
				0.46	4.55		
				0.34	4.5		
				0.26	4.3 4.4		
				0.125	4.1		
				0.063	3.9		
				0.031	3.9		
				0.0167	4.4		
				0.0078	4.8		
				0.0039	No ppt.		
				0.0	" "		

1 cc. of solutions of gelatin B in water (10 per cent in the case of HCl and 7 per cent in the cases of $AlCl_3$ and $La_2(NO_3)_6$) was added to 5 cc. of the solutions of $AlCl_3$, HCl, and $La_2(NO_3)_6$, diluting them to the concentrations noted in the first column in each experiment. The second column of each experiment records the number of cc. of NaCl or $NaNO_3$ solution which had to be added in order to precipitate the gelatin. All the experiments were performed at room temperature. All give curves similar to that for HCl and NaCl which is plotted in Fig. 4.

observing the amount of the precipitate formed. Since the precipitation is always by the oppositely charged ion, the effect of

small concentrations of NaOH in preventing precipitation is to be expected. Whatever the explanation of the favorable effect of higher concentrations of NaOH, this observation is at least in

TABLE V.

The Effect of HCl, NaOH, and $AlCl_3$ on the Precipitation of Gelatin by Sodium Sulfate and Citrate.

Precipitation by Na_2SO_4 of gelatin plus NaOH.		Precipitation by Na_2SO_4 of gelatin plus HCl.		Precipitation by Na_2SO_4 of gelatin plus $AlCl_3$.		Precipitation by Na_3 citrate of gelatin plus NaOH.	
Concentration of NaOH in gelatin.	Na_2SO_4 1 M to precipitate.	Concentration of HCl in gelatin.	Na_2SO_4 1 M to precipitate.	Concentration of $AlCl_3$ in gelatin.	Na_2SO_4 1 M to precipitate.	Concentration of NaOH in gelatin.	Na_3 citrate 1 M to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.
0.687	30	2.5	8.9	1.55	2.8	4.9	No ppt.
0.34	19	1.25	4.8	1.0	4.6	2.45	24
0.17	11.1	0.625	3.5	0.5	6.0	1.27	12
0.085	7.8	0.312	3.3	0.25	7.1	0.64	10
0.043	6.7	0.156	3.1	0.125	6.3	0.32	8
0.021	6.7	0.078	3.0	0.0625	6.6	0.16	8
0.01	8.1	0.039	3.0	0.0312	6.6	0.08	6
0.005	8.6	0.0195	3.1	0.0156	6.9	0.04	12
0.0027	8.7	0.0097	3.6	0.0078	8.0	0.02	11
0.0	7.3	0.0048	4.4	0.0039	8.6	0.01	11
		0.0024	5.1	0.00195	10.7	0.0	11
		0.0	7.2	0.00096	10.8		
				0.0	12.35		

In each experiment the first column shows the concentration of the electrolyte in a 5 cc. sample. In the case of Na_2SO_4 versus HCl and Na_2SO_4 versus NaOH the electrolyte was made up in a 3 per cent solution of gelatin. In the other two experiments, 1 cc. of gelatin solution (5 per cent for Na_2SO_4 versus $AlCl_3$ and 7 per cent for Na_3 citrate versus NaOH) was added to the 5 cc. sample of NaOH or $AlCl_3$ before the precipitant was added. The second column in each experiment records the number of cc. of 1 M Na_2SO_4 or Na_3 citrate which had to be added to the gelatin-electrolyte mixture to cause precipitation. In the experiments with HCl and $AlCl_3$ the proportions of Na:H and Na: Al at the approximate turning points of the curves are 100:1. All four experiments were done at room temperature except the first which was done at 30°C. The results of the first two are plotted in Fig. 5.

agreement with a previous observation of the writer's[11] that the addition of small amounts of NaOH to gelatin containing Na_3 citrate increases the amount of alcohol necessary for pre-

cipitation, while higher concentrations of NaOH decrease this amount. It should also be mentioned that Chick and Martin[1] found, in studying the precipitation of egg albumin, that small amounts of acid and alkali hindered the precipitation by $CaCl_2$ and Na_2SO_4 respectively, but that larger amounts favored precipitation. As $CaCl_2$ does not precipitate gelatin, this part of Chick and Martin's observation could not be confirmed.

The effect of an increase in temperature on the precipitation by NaCl and Na_2SO_4 is striking. An increase in temperature decreases the amount of NaCl necessary to precipitate except in the presence of $Cu(NO_3)_2$ and HNO_3, 0.5 M and 0.05 M, respectively (or stronger), where an increase of temperature causes an increase in the amount of NaCl necessary for precipitation. On the other hand the precipitate of gelatin obtained by using Na_2SO_4 alone is dissolved by an increase in temperature. An examination of the literature shows merely confusion on this point. Mellanby[14] reports that the temperature coefficient of the precipitation of serum proteins by $(NH_4)_2SO_4$ is negative between 0–40°C. and so small as to be unimportant. Lewith[23], on the other hand, finds that a rise in temperature assists the precipitation of ox serum proteins by $(NH_4)_2SO_4$. An observation of Chick and Martin shows that a rise in temperature assisted the precipitation of egg albumin by $(NH_4)_2SO_4$ in alkaline solutions, but in a 10^{-5} normal acid solution the reverse was true above 9°C.

SUMMARY.

Acids, and salts in which the effect of the cation predominates, assist the precipitation of gelatin by NaCl, while alkalies, and salts with predominant anions, hinder it.

Acid favors the precipitation of gelatin by Na_2SO_4. Alkalies hinder it in low concentrations but assist it in higher concentrations, until the gelatin is hydrolyzed by the alkali.

[23] Lewith, S., *Arch. exp. Path. u. Pharmakol.*, 1888, xxiv, 1.

STIZOLOBIN, THE GLOBULIN OF THE CHINESE VELVET BEAN, STIZOLOBIUM NIVEUM.*

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Velvet beans have recently become an important crop in the United States. It is estimated that during the year 1917 more than five million acres were planted in the South. This is an increase of more than 100 per cent over the previous year. Some fifteen species of velvet beans have been grown in the United States. The beans are planted between corn and since their early growth is rather slow they interfere but little with the growth of the corn. The yield of shelled beans is from 10 to 20 bushels per acre.

Most of the velvet beans grown are fed to cattle. In many cases the animals are turned into the fields and eat both the plants and beans. The beans and pods are sometimes ground together and fed as velvet bean meal and pod meal. Cattle thrive well on this meal and it is reported to be an excellent feed for milch cows. In some cases the whole beans are soaked before feeding. This method of feeding has proved very satisfactory in many sections of the South where the beans are not grazed down in the field.

The seed of the Chinese velvet bean is gray in color and about the size and shape of the lima bean. The beans used in this investigation were furnished by the Bureau of Plant Industry. They contained 26.43 per cent of protein ($N \times 6.25$).

The principal protein extracted from velvet bean meal by means of a sodium chloride solution is a globulin which we have named *stizolobin*. This was obtained by dialyzing the sodium chloride

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extracts and was also precipitated from the extract by means of ammonium sulfate. It is probable that only one globulin is extracted by means of sodium chloride solutions since the only protein which precipitated began to appear at 0.4 of saturation with ammonium sulfate and ceased to precipitate when 0.5 of saturation was reached. Protein preparations were also obtained by heating the sodium chloride extract of velvet bean meal at 105°C. The protein thus coagulated was probably nearly all globulin, since its ultimate composition and nitrogen distribution as determined by the Van Slyke method agreed well with the results of analyses made on the globulin obtained by precipitation by dialysis or with ammonium sulfate.

The averages of the percentages obtained for the basic amino-acids by the Van Slyke method on proteins prepared by dialysis, by precipitation with ammonium sulfate, and by coagulation were as follows:

Cystine	1.20
Arginine	6.72
Histidine	2.65
Lysine	8.27

The most striking result here is the high percentage of lysine. In order to check this value, amino nitrogen determinations were made on the unhydrolyzed protein since it has been shown (1) that this amino nitrogen is equal to one-half of the lysine nitrogen in a number of proteins. This is based on the assumption that the α-amino group in lysine is in peptide union, while the ω-amino group is free and is therefore the only amino group in a protein which reacts with nitrous acid. Amino nitrogen determinations were made in triplicate on stizolobin, and the percentages of amino nitrogen obtained were 4.98, 4.81, and 4.78, or an average of 4.86 per cent. This is in good agreement with one-half of the lysine nitrogen which was found to be 5.01 per cent (see Table VII).

Stizolobin also gave a strong qualitative test for tryptophane.

EXPERIMENTAL.

Preliminary Experiments.—The shelled beans were first crushed in a power-driven meat chopper and then ground to a meal. This meal was used for all the experiments described in this paper.

Nitrogen determinations on this meal indicated the presence of 26.43 per cent of protein (N × 6.25). Extraction experiments showed that the maximum quantity of protein was extracted by a 5 per cent sodium chloride solution which gave 22.54 per cent of protein. Table I gives the percentages of protein extracted by various solvents.

In isolating the proteins described in this paper, 5 per cent solution of sodium chloride in distilled water was used for extracting the protein.

Globulin Extracted by Sodium Chloride and Precipitated by Dialysis.—Extracts were made using 5 cc. of solvent to 1 gm. of meal. The quantity of meal used for a preparation was usually 0.5 to 1

TABLE I.

Preliminary Extraction Experiments.

Solvent to 1 gm. of meal.	Solvent.	Protein extracted (N × 6.25).
cc.		*per cent*
5	5 per cent NaCl.	22.54
10	5 " " "	22.30
10	2.5 " " $MgSO_4$.	21.63
25	5 " " NaCl.	21.37
25	2 " " "	20.82
25	10 " " "	20.56
25	15 " " "	18.81
25	20 " " "	17.25
25	Distilled water.	15.28

kilo. The meal was mixed with the solvent and thoroughly stirred to break up all lumps. The mixture was allowed to stand at room temperature for 4 hours, with occasional stirring, and then passed through a peanut grinder to crush the swollen cells. To the thin paste thus obtained was added enough filter paper scrap to make a thick pulp. This was placed in muslin sacks and the liquid pressed out in a powerful press. The turbid solution obtained was filtered through paper pulp on a Buchner funnel. This clear extract was dialyzed in parchment bags suspended in running water until practically free from chlorides. The last traces of chlorides were removed from the protein by washing with distilled water and precipitating the protein by means of a centrifuge. The protein thus obtained was dehydrated, by

suspending it for 12 hours in absolute alcohol which was then decanted after precipitating the protein by centrifugation. It was next suspended for 12 hours in absolute ether and filtered off on a Buchner funnel using a hardened filter. Most of the ether adhering to the protein was removed over sulfuric acid in a vacuum desiccator. The protein was then dried by gradually heating to 110°C. in a vacuum oven. It was ground in a mortar and was thus obtained as a gray, amorphous powder. The yield based on the weight of the meal used was 7 per cent. Three preparations made in this manner gave the following analyses calculated on a moisture-free basis.

TABLE II.

Globulin Precipitated by Dialysis.

	Preparation I.				Preparation II.				Preparation III.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C.....	51.87	51.81	51.84	52.47	51.93	52.14	52.04	52.80	51.78	51.75	51.77	52.80
H.....	7.05	7.06	7.06	7.15	6.89	6.94	6.92	7.01	6.91	6.73	6.82	6.96
N.....	15.99	16.04	16.02	16.16	16.03	16.07	16.05	16.28	15.93	15.87	15.90	16.22
S.....	0.70	—	0.70	0.70	0.73	—	0.73	0.73	0.68	—	0.68	0.69
O*....				23.52				23.18				23.33
Ash..	1.11		1.11		1.33		1.33		1.73		1.73	

* By difference.

Globulin Extracted by Sodium Chloride and Precipitated by Ammonium Sulfate.—Sodium chloride extracts were prepared as described above and the clear solution was made from 0.5 to 0.9 saturated with solid ammonium sulfate. Practically all of the globulin which precipitated came down when the solution was 0.5 saturated. At 0.4 of saturation only a faint turbidity was obtained and between 0.5 and 0.9 only a slight cloud appeared. The precipitated protein rapidly assumed a flocculent form leaving a clear supernatant liquid. It was collected on a folded filter and redissolved in a small volume of distilled water since enough ammonium sulfate adhered to effect solution. The slightly turbid solution was filtered clear through paper pulp and the filtrate was dialyzed until free from sulfates. The globulin so obtained was

dehydrated with absolute alcohol and ether as previously described. The yield was 3 per cent, based on the weight of the meal used. Analyses of four such preparations are given in Table III.

TABLE III.

Globulin Precipitated by Ammonium Sulfate.

	Preparation IV.				Preparation V.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.87	52.68	52.78	53.55	52.60	52.78	52.69	53.13
H	6.83	6.89	6.86	6.96	7.09	7.16	7.13	7.18
N	16.12	16.23	16.18	16.40	16.19	16.34	16.22	16.40
S	—	0.57	0.57	0.58	—	0.62	0.62	0.62
O*				22.51				22.67
Ash	1.32		1.32		0.74		0.74	

	Preparation VI.				Preparation VII.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.68	52.55	52.62	53.20	52.68	52.77	52.73	53.27
H	6.97	—	6.97	7.05	6.94	—	6.94	7.02
N	16.40	16.36	16.38	16.56	16.15	16.15	16.15	16.31
S	—	0.61	0.61	0.61	—	0.64	0.64	0.64
O*				22.58				22.76
Ash	1.03		1.03		0.92		0.92	

* By difference.

A summary of the analyses obtained from seven preparations is given in Table IV. The first three were made by direct dialysis of sodium chloride extracts of velvet bean meal while the remaining four were first precipitated with ammonium sulfate.

Protein Extracted with Sodium Chloride Solution and Coagulated by Heating.—Several preparations of velvet bean protein were made by heating sodium chloride extracts of the meal at 105°C. until no more protein coagulated. The coagulum thus obtained was collected on a folded filter. It was suspended in distilled water and washed by decantation until the washings were free

from chlorides. It was then dehydrated in the usual manner with absolute alcohol and ether. The yield of coagulated protein was 13 per cent, based on the weight of meal used. Analyses of three such preparations are given in Table V.

Distribution of the Nitrogen in Velvet Bean Protein.—The proteins obtained by direct dialysis, by precipitation with ammonium sulfate, and by coagulation were analyzed by Hausmann's modified

TABLE IV.

Summary of Analyses of Velvet Bean Globulin. Ash-Free Basis.

	Preparation No.							
	I	II	III	IV	V	VI	VII	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.47	52.80	52.80	53.55	53.13	53.20	53.27	53.03
H	7.15	7.01	6.96	6.96	7.18	7.05	7.02	7.05
N	16.16	16.28	16.22	16.40	16.40	16.56	16.31	16.33
S	0.70	0.73	0.69	0.58	0.62	0.61	0.64	0.65
O*	23.52	23.18	23.33	22.51	22.67	22.58	22.76	22.94

* By difference.

TABLE V.

Protein Obtained by Coagulation.

	Preparation VIII.				Preparation IX.				Preparation X.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.60	52.83	52.72	53.59	52.08	52.30	52.19	53.01	51.73	51.76	51.75	53.03
H	6.85	6.62	6.74	6.84	6.72	6.67	6.70	6.81	6.81	6.78	6.80	6.97
N	15.65	15.73	15.69	15.94	15.75	15.76	15.76	16.01	15.48	15.50	15.49	15.87
S	0.81	—	0.81	0.82	0.74	—	0.74	0.75	0.78	—	0.78	0.78
O				22.81				23.42				23.35
Ash	1.38		1.38		1.40		1.40		2.24		2.24	

method (2) in order to determine the distribution of the nitrogen. The results of these analyses are given in Table VI.

Analysis of Velvet Bean Protein by the Van Slyke Method.—The globulin precipitated by direct dialysis was analyzed by using duplicate 3 gm. samples, each equivalent to 2.7225 gm. of moisture- and ash-free protein, containing 16.28 per cent of nitrogen. Each

TABLE VI.

N	Globulin precipitated by dialysis.	Globulin precipitated by ammonium sulfate.	Protein obtained by coagulation.
	per cent	*per cent*	*per cent*
Amide	1.55	1.73	1.48
Humin	0.18	0.38	0.34
Basic	3.73	3.95	3.45
Non-basic	10.62	10.34	10.73
Total	16.08	16.40	16.00

TABLE VII.

Analysis of Globulin Precipitated by Dialysis. Van Slyke Method. Total Nitrogen Corrected for Solubility of Bases (4).

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0470	0.0461	10.61	10.41	10.51
Humin N adsorbed by lime	0.0051	0.0059	1.17	1.33	1.25
Humin N in amyl alcohol extract	0.0005	0.0004	0.13	0.08	0.11
Cystine N	0.0041	0.0031	0.93	0.70	0.82
Arginine N	0.0636	0.0616	14.35	13.90	14.13
Histidine N	0.0183	0.0154	4.13	3.49	3.81
Lysine N	0.0441	0.0447	9.95	10.09	10.02
Amino N of filtrate	0.2472	0.2506	55.77	56.54	56.16
Non-amino N of filtrate	0.0134	0.0139	3.03	3.14	3.09
Total N regained	0.4433	0.4417	100.07	99.68	99.90

TABLE VIII.

Basic Amino-Acids in Globulin Precipitated by Dialysis.

	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.29	0.97	1.13
Arginine	7.25	7.02	7.14
Histidine	2.48	2.09	2.27
Lysine	8.45	8.57	8.51

sample, therefore, contained 0.4432 gm. of nitrogen. The protein was dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solution for 36 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (3). The results are given in Tables VII and VIII.

The globulin precipitated by ammonium sulfate was analyzed in the manner just described. The protein used contained 16.31

TABLE IX.

Analysis of Globulin Precipitated by Ammonium Sulfate (Van Slyke Method). Total Nitrogen Corrected for Solubility of Bases (4).

	I	
	gm.	*per cent*
Amide N	0.0403	10.67
Humin N adsorbed by lime	0.0045	1.19
Humin N in amyl alcohol extract	0.0003	0.07
Cystine N	0.0035	0.93
Arginine N	0.0498	13.19
Histidine N	0.0168	4.45
Lysine N	0.0351	9.30
Amino N of filtrate	0.2179	57.71
Non-amino N of filtrate	0.0118	3.13
Total N regained	0.3800	100.64

TABLE X.

Basic Amino-Acids in Globulin Precipitated by Ammonium Sulfate.

	per cent
Cystine	1.29
Arginine	6.68
Histidine	2.74
Lysine	7.88

per cent of nitrogen. The sample weighed 2.6 gm. and was equivalent to 2.3150 gm. of moisture- and ash-free protein. It contained 0.3776 gm. of nitrogen. The results are given in Tables IX and X.

The protein obtained by coagulation was also analyzed by the Van Slyke method. Duplicate 3 gm. samples were used, each equivalent to 2.7144 gm. of moisture- and ash-free protein and

TABLE XI.

Analysis of Protein Obtained by Coagulation (Van Slyke Method).

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0468	0.0428	10.83	9.92	10.38
Humin N adsorbed by lime	0.0063	0.0060	1.46	1.40	1.43
Humin N in amyl alcohol extract	0.0001	0.0004	0.03	0.10	0.07
Cystine N	0.0040	0.0035	0.93	0.81	0.87
Arginine N	0.0543	0.0566	12.55	13.11	12.83
Histidine N	0.0219	0.0154	5.07	3.57	4.32
Lysine N	0.0436	0.0445	10.10	10.31	10.21
Amino N of filtrate	0.2497	0.2531	57.84	58.63	58.24
Non-amino N of filtrate	0.0126	0.0109	2.92	2.52	2.72
Total N regained.*	0.4393	0.4332	101.73	100.37	101.07

*Total nitrogen corrected for solubility of bases (4).

TABLE XII.

Basic Amino-Acids in Protein Obtained by Coagulation.

	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.26	1.10	1.18
Arginine	6.21	6.47	6.34
Histidine	3.04	2.14	2.64
Lysine	8.34	8.52	8.43

TABLE XIII.

Free Amino Nitrogen of Velvet Bean Globulin Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N. Van Slyke method.
mg.	*cc.*	*mm.*	*°C.*	*mg.*	*per cent*	*per cent*
6.36	0.56	771	24	0.317	4.98	5.01*
6.36	0.54	771	24	0.306	4.81	
16.71	1.41	768	23	0.799	4.78	

* From Table VII.

containing 15.87 per cent or 0.4317 gm. of nitrogen. The results are recorded in Tables XI and XII.

Determination of the Free Amino Nitrogen in Velvet Bean Globulin.—The solutions used for these determinations were made by dissolving the freshly prepared protein in a 5 per cent sodium chloride solution. The total nitrogen in 10 cc. of this solution was then found. The free amino nitrogen was determined in the usual manner (1), using 2 cc. of solution in the Van Slyke micro-apparatus. Caprylic alcohol was used to prevent foaming and corrections were made for the reagents used. The results obtained are given in Table XIII.

SUMMARY.

1. The globulin of the Chinese velvet bean has been isolated and analyzed. This protein has been named *stizolobin*.

2. The basic amino-acids in stizolobin have been determined by the method of Van Slyke.

3. Analyses indicate that stizolobin contains all the basic amino-acids known to exist in proteins. The lysine content is 8.27 per cent of the protein. Tryptophane is also present.

4. The free amino nitrogen in stizolobin has been determined and has been found equal to very nearly one-half of the lysine nitrogen as determined by the Van Slyke method.

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THE GLOBULIN OF BUCKWHEAT, FAGOPYRUM FAGOPYRUM.

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As far as we have been able to ascertain, Ritthausen[1] is the only investigator who has published the results of any research on the protein of buckwheat. His data appeared in 1872 in a collection of researches dealing with the proteins of various kinds of seeds. Ritthausen used an especially prepared buckwheat meal for his experiments and states that his best material contained a considerable proportion of finely ground bran. This meal contained 10.44 per cent of protein.

The material used for our experiments consisted of buckwheat flour and was bought under a Government contract. It was examined by the Pharmacognosy Laboratory of this Bureau and declared to be free from adulterants. Two lots of flour were used and these contained respectively 6.5 and 7.8 per cent of protein ($N \times 6.25$). This is considerably less than was contained in the meal used by Ritthausen. This difference is probably due to the fact that our flour was more highly milled than Ritthausen's meal, and therefore contained less bran and more starch.

We tried to extract a protein from buckwheat flour by warming it in 70 per cent alcohol, by volume, but an analysis showed that only traces of nitrogen were thus extracted. This result is in agreement with Ritthausen's experience. He came to the conclusion that buckwheat meal contains but little if any alcohol-soluble protein.

Ritthausen states that it was difficult to obtain protein preparations in a relative state of purity from buckwheat meal because

[1] Ritthausen, H., Die Eiweisskörper der Getreidearten, Hülsenfrüchte und Ölsamen, Bonn, 1872, 150.

the extracts are of a slimy consistency due to the presence of gum. We encountered similar difficulties.

Ritthausen published the analyses of 5 different preparations of buckwheat protein. Four of these were made by extracting the meal with very dilute potassium hydroxide solutions and precipitating the protein with acetic acid. In the case of the fifth preparation the meal was extracted with water and acetic acid was used to precipitate the protein.

Our most successful preparations of buckwheat globulin were obtained by extracting the flour with 5 or 10 per cent sodium chloride solutions, precipitating the protein with ammonium sulfate, redissolving it in water, and dialyzing the solution until salts were removed. The yield obtained by this procedure was small and represented only about 20 per cent of the nitrogen present in the flour. The composition of the globulin thus obtained together with the results published by Ritthausen are given below.

	Buckwheat globulin. Average of eight preparations.	Average of five preparations by Ritthausen.
C	51.69	50.86
H	6.90	6.95
N	17.44	16.08
S	1.16	1.11
O*	22.81	25.00

* By difference.

The nitrogen in the buckwheat globulin was distributed as follows:

Humin	0.19
Amide	1.78
Basic	5.29
Non-basic	10.44
Total	17.70

The results of analyses by Van Slyke's method indicate that the globulin contains arginine, histidine, lysine, and cystine. A qualitative test for tryptophane was also obtained. The most striking result here is the high percentage of lysine.

The percentage of basic amino-acids in the globulin of buckwheat is:

Arginine	12.97
Histidine	0.59
Lysine	7.90
Cystine	1.00

The high percentage of basic amino-acids in buckwheat flour is of importance in view of the fact that buckwheat flour is frequently used together with wheat flour. A mixture of these two flours would have a lower protein content than wheat flour. The proteins of the mixed flour would, however, probably be more efficient since the percentage of basic amino-acids would be higher than in wheat flour alone.

EXPERIMENTAL.

Preliminary Experiments on the Buckwheat Flour.—Distilled water at room temperature extracted 2.5 per cent of protein from the flour, while 5 or 10 per cent sodium chloride solutions extracted 4.5 per cent of protein. A 5 per cent solution of magnesium sulfate extracted 3.8 per cent of protein. A 0.2 per cent potassium hydroxide solution extracted 5.3 per cent of protein.

Globulin Extracted by Sodium Chloride Solution and Precipitated by Dialysis.—A sodium chloride extract of buckwheat flour was made using 875 gm. of flour and 2,500 cc. of a 5 per cent sodium chloride solution. After standing over night in a refrigerator, this mixture was filtered through paper pulp on a Buchner funnel. In this manner 1,500 cc. of filtrate were obtained. The extract was very slimy and difficult to filter. The filtrate was dialyzed against running tap water for 16 days. The globulin was precipitated as a compact mass. This was washed free from chlorides by means of distilled water and thrown down by means of the centrifuge after each washing. It was then suspended in absolute alcohol and dehydrated over night. It was finally suspended in absolute ether to remove the alcohol. After filtering off the ether, the globulin was placed in a vacuum desiccator over sulfuric acid to remove the ether and dried gradually to 100°C. in a vacuum oven. It was finally dried to constant weight in an air oven at 110°C. The yield was 1 per cent based on the volume of filtrate obtained. By this procedure the globulin was obtained

as a gray powder which contained 2.04 per cent of ash. The average results of duplicate analyses were C, 51.49, H, 6.95, N, 16.87 per cent.

Globulin Precipitated by Ammonium Sulfate.—2 kilos of buckwheat flour were ground with 5 liters of a 5 per cent sodium chloride solution and the mixture was placed in a refrigerator over night. It was then filtered through paper pulp on a Buchner funnel. The volume of liquid obtained was usually about 2,250 cc. Solid ammonium sulfate was added to this filtrate to 0.9 of saturation. After standing over night the precipitated protein was filtered off on paper pulp. The protein and paper pulp were then macerated in a small volume of distilled water. Enough ammonium sulfate remained with the protein and pulp to effect solution. After filtering, the clear filtrate was dialyzed against running water for 10 days. The precipitated globulin was then treated as previously described. It was finally obtained in the form of an almost white powder. The yield was 1 per cent based on the volume of liquid obtained from the extraction with sodium chloride. Analyses of eight different preparations obtained by this method are given below.

TABLE.

Analyses of Buckwheat Globulins.

	Preparation I.				Preparation II.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
C	50.86	50.83	50.85	51.28	50.94	50.65	50.80	51.26
H	7.16	6.96	7.06	7.12	6.55	6.84	6.75	6.81
N	16.64	16.81	16.73	16.87	17.35	17.40	17.38	17.54
S	1.04		1.04	1.05	1.13		1.13	1.14
O*				23.68				23.25
Ash	0.81		0.81		0.86		0.86	

	Preparation III.				Preparation IV.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
C	51.18	50.95	51.07	51.52	51.28	51.11	51.20	51.66
H	6.83	6.81	6.82	6.88	6.72	6.67	6.70	6.76
N	17.45	17.11	17.28	17.43	17.63	17.61	17.62	17.78
S	1.30		1.30	1.31	1.21		1.21	1.22
O*				22.86				22.58
Ash	0.88		0.88		0.90		0.90	

	Preparation V.				Preparation VI.			
	I	II	Average.	Ash-free.	I	II	Average.	Ash-free.
C	51.60	51.62	51.61	52.13	51.69	51.75	51.72	52.36
H	6.77	6.73	6.75	6.81	6.93	6.93	6.93	7.01
N	17.50	17.40	17.45	17.62	17.14	17.21	17.18	17.40
S	0.97		0.97	0.98	1.18		1.18	1.19
O*				22.46				22.04
Ash	0.95		0.95		1.24		1.24	

	Preparation VII.				Preparation VIII.	
	I	II	Average.	Ash-free.		Ash-free.
C	51.64	51.57	51.61	52.03	50.62	51.28
H	7.01	6.88	6.95	7.01	6.73	6.81
N	17.30	17.58	17.44	17.60	17.06	17.28
S	1.20		1.20	1.21	1.13	1.15
O*				22.15		23.48
Ash	0.82		0.82		1.30	

TABLE.—*Concluded.*

Summary.

Preparation No.	I	II	III	IV	V	VI	VII	VIII	Average.
C	51.28	51.26	51.52	51.66	52.13	52.36	52.03	51.28	51.69
H	7.12	6.81	6.88	6.76	6.81	7.01	7.01	6.81	6.90
N	16.87	17.54	17.43	17.78	17.62	17.40	17.60	17.28	17.44
S	1.05	1.14	1.31	1.22	0.98	1.19	1.21	1.15	1.16
O	23.68	23.25	22.86	22.58	22.46	22.04	22.15	23.48	22.81

* By difference.

Distribution of the Nitrogen in Buckwheat Globulin.—Determinations made by Hausmann's modified method to determine the distribution of the nitrogen in this globulin gave the following results:

N	I	II	Average.
Humin	0.26	0.11	0.19
Amide	1.74	1.81	1.78
Basic	5.01	5.56	5.29
Non-basic	10.69	10.22	10.44
Total	17.70	17.70	17.70

Analysis of Buckwheat Globulin by the Van Slyke Method.—Analyses were made on two different preparations, using 2.4601 and 2.1056 gm. of protein. These samples contained 0.4331 and 0.3727 gm. of nitrogen respectively. They were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method.

Analysis of Buckwheat Globulin, Van Slyke Method.

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0418	0.0350	9.65	9.39	9.52
Humin N	0.0144	0.0130	3.33	3.49	3.42
Arginine N	0.1023	0.0884	23.62	23.72	23.67
Histidine N	0.0049	0.0024	1.13	0.64	0.88
Cystine N	0.0026	0.0027	0.60	0.73	0.67
Lysine N	0.0366	0.0327	8.45	8.77	8.61
Amino N of filtrate	0.2287	0.1881	52.81	50.47	51.64
Non-amino N of filtrate	0.0017	0.0095	0.39	2.55	1.47
Total N regained*	0.4330	0.3718	99.98	99.76	99.88

* Total nitrogen corrected for solubility of bases.

Basic Amino-Acids in Buckwheat Globulins.

Amino-acid.	I	II	Average.
	per cent	*per cent*	*per cent*
Arginine	12.91	13.03	12.97
Histidine	0.75	0.43	0.59
Lysine	7.73	8.07	7.90
Cystine	0.90	1.10	1.00

SUMMARY.

1. The globulin of buckwheat has been isolated and analyzed. Results indicate that it contains the basic amino-acids, arginine, histidine, lysine, and cystine. It also contains tryptophane.

2. Results indicate that buckwheat globulin contains a relatively high percentage of lysine.

THE PRODUCTION OF GLYCOSURIA BY ZINC SALTS.*

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INTRODUCTION.

The effects of zinc have been studied from different points of view by various investigators. While considerable attention was directed toward its general biological action some observations on its behavior in the body have been conducted either with a view to the therapeutic value of some of its compounds or on account of its importance in hygiene, due to the deleterious effects caused by zinc in numerous industries and because of its occurrence in food products. As the amount of zinc found in food may be quite large, considerable interest attaches to the changes which it produces in the body. Accordingly new studies on its action were undertaken, the present investigation being the first of a series of reports on the subject. Although some of the effects produced by zinc and its salts have already been established by experiments on animals and in cases of poisoning in man, the observations were confined almost entirely to changes in function or structure of some organs, no systematic inquiries having been reported on the alteration of metabolic processes caused by the metal or any of its compounds. The only record of an attempt at such a study which we could find after a diligent search of the literature, was a casual observation of d'Amore, Falcone, and Maramaldi.[1] According to these investigators considerable

*The senior author disclaims all responsibility for previous publications on this subject purporting to come from this laboratory and involving quantitative determinations of sugar in the blood and in the urine.

[1] d'Amore, L., Falcone, C., and Maramaldi, L., *Compt. rend. Soc. biol.*, 1892, iv, 335.

amounts of sugar, as well as albumin and casts, were found in the urine of dogs that had been fed 0.5 to 1.0 gm. of zinc daily.

As zinc resembles other heavy metals in the changes it produces in animals, it is of interest to recall in this connection the observations on some of the effects caused by them, particularly the production of glycosuria and the changes of the concentration of sugar in the blood associated with lesions of the kidney.

According to Schroeder[2] glycosuria was observed after small doses of mercuric chloride. This was corroborated by Kissel,[3] who found about 0.5 per cent of sugar in the urine, and reported that the concentration of sugar in the blood was normal or below. That glycosuria may be caused by sublimate was also shown by Richter,[4] but he also stated that it is associated with hyperglycemia, the blood sugar being increased 60 to 100 per cent in some animals. After intravenous injection of 3 mg. of bichloride of mercury into rabbits, Frank[5] found 0.25 per cent to 1.03 per cent of sugar in the urine, the amounts present in the blood being normal. Luzzatto[6] reported 0.5 per cent to 1.4 per cent sugar in the urine and 0.147 per cent sugar in the blood after the subcutaneous injection of corrosive sublimate into rabbits. Similar studies on this subject were also made by Graf,[7] and according to Lépine[8] glycosuria may occur in man after corrosive sublimate.

Numerous investigations were carried out with the salts of uranium. Since its discovery by LeConte,[9] glycosuria produced by uranium salts has been reported by Chittenden and Lambert,[10] Woroshilsky,[11] Meyner,[12] Lépine and Boulud,[13] Fleckseder,[14] Pollak,[15] Frank,[5] and Luzzatto.[6] According to Frank and Luzzatto the amounts of sugar in the urine varied between 0.02 and 0.75 per cent, and glycosuria was also found in the urine of fasting animals, but the amount of sugar was less and appeared later than in well fed animals. Its duration, as shown by Frank in experiments on dogs, was 8 to 11 days after a single injection of the salts. Reports of

[2] Schroeder, H., Dissertation, Würzburg, 1893.
[3] Kissel, O., Dissertation, Würzburg, 1894.
[4] Richter, P. F., *Deutsch. med. Woch.*, 1899, xxv, 840.
[5] Frank, E., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 387.
[6] Luzzatto, R., *Z. exp. Path. u. Therap.*, 1914, xvi, 18.
[7] Graf, E., Dissertation, Würzburg, 1895.
[8] Lépine, R., *Arch. med. exp. et anat. path.*, 1903, xv, 129.
[9] LeConte, *Gaz. méd. Paris*, 1854, ix, 196.
[10] Chittenden, R. H., and Lambert, A., *Z. Biol.*, 1889, xxv, 513.
[11] Woroshilsky, J., Dissertation, Dorpat, 1889.
[12] Meyner, H., Dissertation, Würzburg, 1898.
[13] Lépine, R., and Boulud, *Rev. méd.* 1904, xxiv, 1.
[14] Fleckseder, R., *Arch. exp. Path. u. Pharmakol.*, 1906–07, lvi, 54.
[15] Pollak, L., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 376.

the concentration of sugar in the blood were conflicting, some having observed hyperglycemia, others normal or subnormal amounts.

Chromate glycosuria has also been reported by a number of observers. To Veron[16] belongs the credit of first calling attention to the production of glycosuria by the salts of chromium. But more extensive studies of the subject were made later by Kóssa,[17] who determined the amount of sugar in the urine as well as in the blood. According to this writer no hyperglycemia was observed in animals poisoned with chromate. This was contradicted, however, by other investigators, some of whom obtained values that were either higher or much lower than those reported by Kóssa. The amounts of sugar in the urine did not exceed 1.2 per cent, the glycosuria lasting for weeks after a single injection. This subject was also studied by Blanck,[18] Pollak,[15] Frank,[5] and later by Luzzatto,[6] all of whom found sugar in the urine, but disagreed as to the amounts present in the blood after the administration of chromium salts. According to Pollak, the concentration of sugar in the blood of rabbits that received chromate subcutaneously may reach about 0.254 per cent. The amounts he found in the urine were 0.28 and 1.3 per cent. Luzzatto stated that glycosuria and albuminuria occurred after potassium chromate. He reported 2 per cent of sugar in the urine in one experiment and 1 per cent in another, and stated that sugar was observed in the urine of well fed and of fasting animals. According to Luzzatto,[6] tellurium salts, especially sodium tellurate, may also cause glycosuria and albuminuria in animals, the amount of sugar in the urine usually being 0.3 to 0.5 per cent and reaching sometimes 1 per cent. The glycosuria, which may last 5 days, sets in within 48 hours after subcutaneous injection, albuminuria appearing before the glycosuria. It was observed by him in well nourished animals and in those that have fasted several days. The concentration of sugar in the blood, according to Luzzatto, varied between 0.075 and 0.095 per cent.

Glycosuria was also reported by Filippi[19] in experiments with selenium.

Methods.

The experiments were carried out on rabbits, cats, and dogs. The zinc salts were administered in the form of malate or acetate, the former when introduced intravenously, subcutaneously, or intramuscularly, the latter when given by mouth. Blood sugar was determined by the method of Lewis and Benedict,[20] the blood for the analyses being obtained from the heart by a hypodermic needle which was introduced into it through

[16] Veron, Thèse de Paris, 1885, cited by Lépine, Le diabète sucré, Paris, 1909.

[17] Kóssa, J., *Arch. ges. Physiol.*, 1902, lxxxviii, 627.

[18] Blanck, S., *Med. Klin.*, 1905, i, 1144.

[19] Filippi, E., *Sperimentale*, 1913, lxvii, 565.

[20] Lewis, R. C., and Benedict, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 57.

the chest wall. For the determination of the sugar in the urine, Allihn's method was employed throughout this investigation.

The amount of albumin in the urine was roughly determined by weighing the dried precipitate obtained by coagulation. The following symbols were used to indicate the approximate quantities present:

+	1 mg. albumin per cc. urine.
++	1 to 2 mg. albumin per cc. urine.
+++	2 " 3 " " " " " etc.

Appearance of Glycosuria and Albuminuria after the Administration of Zinc Malate and Zinc Acetate.

The intravenous injection into rabbits of 9 to 10 mg. of zinc per kilo in the form of malate was followed by albuminuria associated with glycosuria and hyperglycemia. The quantity of sugar in the urine varied from mere traces to about 0.3 per cent, but in one rabbit the urine contained 1.6 per cent sugar. Glycosuria was observed in some experiments 6 hours after the injection of zinc malate, but its appearance was sometimes delayed until the 2nd, 3rd, or 4th day, and in two rabbits it was first noticed on the 8th day after zinc was given. Its duration also varied in different experiments, being transitory in some cases, as sugar was found on 1 day only, while the urine of other rabbits contained sugar for 2, 3, and 4 days in succession. The albuminuria usually preceded the glycosuria, was often very marked, and bore no relation to the amount of sugar in the urine. In some cases it appeared at the same time and in one experiment the glycosuria, on the contrary, preceded the albuminuria. Mention might be made in this connection that glycosuria was frequently absent in rabbits that had been receiving carrots at the time of the injection of zinc while the amounts of albumin in the urine were sometimes negligible and soon disappeared.

Zinc salts administered subcutaneously or by mouth likewise produced glycosuria and albuminuria, but much larger doses were required. 100 mg. of zinc per kilo, when injected subcutaneously, were followed by marked glycosuria in some rabbits while doses of 50 to 75 mg. of zinc per kilo were much less effective. It is important to note that in these experiments also the albuminuria was more pronounced and was likewise more persistent than the glycosuria. When zinc was given by mouth, glycosuria was less frequent, although large amounts of the metal, 300 mg. per kilo,

which were fed in the form of acetate, were administered. As observed in experiments by subcutaneous injection, albuminuria was constant and was more marked than the glycosuria. In some experiments 20 gm. of sucrose per kilo were dissolved and given by mouth. The administration of zinc acetate later to these animals evoked glycosuria more readily than in those which had not received sugar. The presence of dextrose in the urine was usually detected on the day following the administration of the salts. In some instances its appearance was delayed 5 or 7 days. In all of our experiments in which the zinc salts were given subcutaneously or by mouth, glycosuria was transitory as it was never found in the urine on subsequent days.

Glycosuria was likewise observed after the subcutaneous injection into cats of 25 to 100 mg. of zinc in the form of malate. This was noticed in a considerable proportion of experiments, but not in all. The amounts of sugar were much larger, however, than in the urine of rabbits that were similarly treated, for after the injection of malate into cats the urine contained 0.5 to 2.72 per cent of glucose. Sugar was detected in the urine in two experiments on the day following the injection, in a third within 3 days, in a fourth in 5 days. The duration of glycosuria was 1 day in all cases except one in which the urine contained sugar on 4 successive days. In all cases traces, or small amounts of albumin appeared in the urine within 1 to 3 days, but the amount was considerably increased a few days later.

Experiments on dogs were made with 15 to 26 mg. of zinc per kilo which was injected intramuscularly. In two experiments the albuminuria was very moderate in degree but in the third considerable amounts of albumin were present. The test for sugar in the urine was positive in two experiments, but no quantitative determinations were made. The duration of life was 1 to 5 days.

Finally, attention may be called to the relation of food and fasting to the glycosuria observed after zinc. In all cases its administration, especially when given intravenously and subcutaneously, was followed by almost immediate loss of appetite. Since the glycosuria frequently persisted more than 1 day, sugar being found in the urine of the 3rd or even of the 4th day, it is evident that it is independent of the nutritional condition of the

TABLE I.

Production of Glycosuria and Albuminuria by Zinc.

Rabbit No.	Diet.	Weight.	Zinc per kilo.	Glucose in urine (maximum amount).	Albumin in urine (maximum amount).	Duration of life.	Remarks.
				Intravenous injections.			
		gm.	*mg.*	*per cent*		*days*	
1,504	Oats.	1,420	9	0.1	++	4	No albumin or sugar before injection. Both present 6 hrs. after injection of zinc malate. 60 cc. of urine obtained in first 24 hours; after this anuria and loss of appetite. Sugar found on 1st, 2nd, and 4th days.
1,517	"	1,455	10	0.3	++	About 2.	190 cc. of water, but no food ingested after zinc malate. 45 cc. of urine 1st day, none the next day.
1,538	"	1,385	10	0.3 on 2nd day.	+++ on 2nd day.	3	Complete loss of appetite; 225 cc. of water ingested after zinc malate. 42 cc. of urine first 24 hours, then anuria.
1,501	Carrots.	2,020	9	+ in 2 days.	++++ in 24 hrs.	2	++ albumin 6 hrs. after injection, but no sugar in urine. Appetite poor; drank 320 cc. of water in the 2 days after zinc malate. Passed 70 cc. urine in first 6 hrs. and 75 cc. during the next 2 days.
1,490	"	1,515	9	None.	++++	2	Appetite fair; drank almost normal amount of water. 50 cc. urine first 6 hours. 70 cc. in the next 2 days. No albumin or sugar before injection.

				Subcutaneous injection.			
1,277	Oats.	1,900	100		++++ in 24 hrs.	8	Albuminuria decreased in severity after 24 hrs. Ascites and gastroenteritis observed at autopsy.
1,278	"	1,975	100	+++ in 24 hrs.	+++		Hydrocele, ascites, and gastroenteritis at autopsy. Glycosuria disappeared after 24 hrs.
1,321	"	1,415	100	+++	+++	5	Albuminuria developed within 24 hrs. Glycosuria was observed on the 1st day after injection of zinc malate.
1,394	"	1,625	75	+	+++	Survived.	Albuminuria noticed within 24 hrs. Albuminuria subsided 2 days later. No sugar found in the urine the following day.
1,386	"	1,510	50	+	Trace.	"	Loss of appetite for about 5 days, but improvement observed later. Sugar found 1 week after injection. Albumin, trace only.
				Administration by mouth.			
1,268	Carrots.	1,900	335	++	++++	Survived.	Zinc given in the form of acetate; 30 to 100 mg. zinc previously received failed to cause albuminuria and glycosuria.
1,156	Oats.	1,755	335	++	++++	7	No albumin or sugar before zinc was injected. 20 gm. sucrose given per kilo in 50 per cent solution 4 days before zinc. Sugar detected in urine 24 hrs. after zinc.

TABLE 1—*Concluded.*

Cat 264. White, Male.

Date.	Weight.	Meat.	Water.	Urine.			Remarks.
				Volume.	Albumin.	Sugar.	
1914	*gm.*	*gm.*	*cc.*	*cc.*			
Nov. 5	2,365				Not examined.	Trace.	Received intramuscular injection of 60 mg. zinc as malate in 0.9 per cent NaCl. Site, femoral region. Time, 11.00 a.m.
" 6		15	190	25	Heavy trace.	1.55 per cent.	
" 7		10	20	28	80 mg. per 100 cc.	Trace.	
" 9		100	120	105	460 mg. per 100 cc.	None.	48 hr. protocol. Epithelial casts.
" 10		100	110	80	+ + + + +	"	
" 11		100	110	90	+ + + +	"	
" 12		100	100	74	+ + + +	"	
" 13		100	60	60	+ + +	"	
" 14	2,290	100	65	50	+ +	"	
" 16		100	140	108	+ +	"	
" 17		100	65	100	+ + +	"	48 hr. protocol. Cat apparently infected
" 18		40	25	50	+ + +	+	Animal chloroformed.

Cat 273. White and Gray. Male.

Nov. 11	2,215				Trace.	None.	Subcutaneous injection in the back of 64 mg. zinc as malate in 0.9 per cent NaCl, 4.30 p.m.
" 12		12	165	30	++	Faint trace.	
" 13		Nothing.	20	58	++	None.	Urine contains small amount of putrid matter in suspension readily filtered out.
" 14	2,135	40	40	30	+++	+	
" 16		Nothing.	30	60	+++++	+	
" 17		"	30	30	+++++	+	
" 18		"	20	40	+++++++	++	Eyes closed with purulent discharge. No sign of infection at site of injection. Chloroformed.

Cat 263. Tiger, Female.

Nov. 4							Injected 60 mg. zinc as malate intramuscularly in femoral region, 2.00 p.m.
" 5, 9.00 a.m.				45	Trace.	2.72 per cent.	
" 5				25	Not tested.	None.	Urine passed between 9.00 a.m. and 2.00 p.m.
" 6		Nothing.	125	55	++	"	Protocol for 24 hrs. with exception of urine which is from 2.00 p.m. to 9.00 p.m.
" 7		"	40	30	396 mg. in 100 cc.	"	
" 9		"	50	65	694 mg. in 100 cc.	"	Epithelial casts in urine. Died at noon.

TABLE II.

Showing Concentration of Sugar in the Blood of Rabbits after Intravenous Injection of Zinc Malate (10 Mg. of Zinc per Kilo).

Rabbit No.	Sex.	Diet.	Glucose per cc. of blood before injection of zinc.	Glucose per cc. of blood following development of glycosuria.	Duration of life after injection of zinc malate.	Remarks.
			mg.	*mg.*	*days*	
1,466	♂	Oats.	1.40 (3 days)* 1.34 (1 day).	1.89 (3 days).†	About 3.	Albuminuria +++, glycosuria +. Died directly after bleeding.
1,469	♂	"	1.60 (5 days). 1.54 (3 ").	2.02 (about 2 days).	" 6.	Highest albumin +++++, glycosuria ++.
1,413	♂	"	1.60 (5 "). 1.57 (1 day).	2.00 (1 day). 4.36 (about 6 days).‡	6	Previous history: albumin ++++, 1.6 per cent sugar.
1,484	♀	Carrots.	1.41 (5 days). 1.39 (1 day).	1.99 (1 day).	2	Highest albumin +++, glycosuria ++.
1,482	♀	"	1.52 (5 days). 1.52 (3 ").	2.31 (3 days).	3	Died by internal bleeding. Highest albumin ++, glycosuria ++.
1,464	♂	"	1.42 (5 "). 1.29 (3 ").	1.72 (1 day).	Complete recovery.	No albumin developed except on 1 day long after Zn injection. Glycosuria lasted about 48 hrs. 16 days after injection the blood sugar content was normal, being 1.395 mg. per cc.
1,473	♂	"	1.42 (3 "). 1.37 (1 day).	2.00 (7 days).	8 to 9 days.	Day following injection a faint (very temporary) glycosuria was noted, but the blood sugar content was normal (1.35 mg. per cc.). Only traces of albumin noted. Glycosuria +++.

1,414	♀	Initial diet, oats followed by period of fasting (48 hrs. before injection). Placed on carrots 2 days after injection.	1.48 (11 days). 1.50 (7 "). 1.63 (5 "). 1.54 (1 day).	1.96 (3 days).	About 8 days.	Prior to carrot feeding albumin +++++. Fell to +++ within 24 hrs. and gradually to ++ glycosuria.

Average amounts of sugar in the blood: Before injection, 1.47 mg. per cc.; after injection, 1.98 mg. per cc.

* Figures in parentheses indicate interval between determination of sugar and injection of zinc.

† Figures in parentheses indicate interval between injection of zinc and determination of sugar.

‡ Excludea from average.

subject, since it may even occur in fasting. This, as will be seen later, is important in throwing light on the nature of the glycosuria produced by zinc.

Concentration of Sugar in the Blood after the Intravenous Injection of Zinc.

In view of the conflicting results obtained in experiments with other heavy metals it was especially interesting to determine whether the glycosuria observed after the administration of the salts of zinc is associated with any change in the sugar content of the blood. Only rabbits in which the blood sugar content showed little variation were chosen for the tests. The results of the experiment given in Table II show that a decided increase in blood sugar followed the intravenous injection of 10 mg. per kilo of zinc in the form of zinc malate, the average increase being approximately 33 per cent. It will be remarked, however, that there was practically no difference in the amounts of blood sugar of rabbits on a diet of oats and those on a diet of carrots.

Since there was loss of appetite after the administration of zinc it was considered advisable to determine also the amount of sugar in the blood in fasting rabbits. Analysis of the blood made after 2 days of fasting, the rabbits having been fed previously oats or carrots, showed that the concentration of sugar may vary between 0.129 and 0.160 per cent.

Influence of Calcium Chloride on Zinc Glycosuria.

The effects of calcium in conditions of abnormal sugar metabolism have been studied by several investigators. Brown[21] reported that calcium chloride prevented the excretion of sugar in the urine in phlorhizin diabetes and in glycosuria produced by sodium chloride. This was corroborated later by Fischer[22] and denied by McGuigan and Brooks,[23] who failed to inhibit sodium chloride glycosuria by means of calcium. But according to Underhill and Closson,[24] glycosuria produced by the intravenous infusion of sodium chloride may be partly or entirely inhibited when calcium chloride is administered simultaneously. These results were corroborated later

[21] Brown, O. H., *Am. J. Physiol.*, 1903–04, x, 378.
[22] Fischer, M. H., *Arch. ges. Physiol.*, 1905, cix, 1.
[23] McGuigan, H., and Brooks, C., *Am. J. Physiol.*, 1907, xviii, 256.
[24] Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905–06, xv, 321.

by Underhill and Kleiner.[25] Jacoby and Eisner[26] caused a marked decrease or a complete inhibition of the excretion of sugar in phlorhizin diabetes in dogs.

The effect of calcium on glycosuria produced by zinc malate was tested in the following experiments, 10 to 15 cc. per kilo of an M/8 calcium chloride solution being injected intravenously immediately before or after the zinc salts, of which 10 mg. per kilo were given in the form of malate. It will be remarked, as shown in Table III, that the amount of sugar in the urine was in some cases much larger than that produced by zinc malate alone. In one of the four experiments a trace only of sugar was present while in two the amounts of sugar found in the urine were 0.4 per cent and 0.5 per cent in 24 hours, and in one case it was 0.2 per cent in 48 hours. Tests for sugar could be obtained on the next day after the injection of calcium and zinc, but none was present after the 4th or 5th day. The appearance of albumin preceded that of the sugar in one experiment (Rabbit 1,516), but in the others both were observed at about the same time.

SUMMARY AND DISCUSSION.

Briefly stated, the essential facts established are as follows: When zinc malate was injected intravenously into rabbits, glycosuria and hyperglycemia appeared; the presence of sugar in the urine as well as its increased amount in the blood being observed in well fed and in fasting animals; when sucrose was fed glycosuria was produced more easily. Glycosuria was also produced by subcutaneous injections and after the administration of zinc malate or acetate *per os* but much larger doses were required. The amount of sugar in the urine was never large, being usually about 0.35 per cent or less, in rabbits, but much larger averages were found in the urine of cats. Albuminuria, which in some cases became very pronounced, was likewise observed after administration of zinc salts, and it appeared either simultaneously with or before the glycosuria and lasted sometimes until the animal died. The duration of the glycosuria was about 1 to 4 days and also appeared irregularly in some experiments. Glycosuria, as

[25] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 395.
[26] Jacoby, M., and Eisner, G., *Ber. klin. Woch.*, 1913, l, 1339.

TABLE III.

Experiments with Zinc Malate and Calcium Chloride.

Rabbit No.	Time.	Albumin.	Sugar.	Oats.	Water.	Urine.	Remarks.
				gm.	*cc.*	*cc.*	
1,516	1st day (18 hrs.).	++	None.	None.	60	85	13 mg. zinc as malate injected intravenously, followed by 13 cc. M/8 $CaCl_2$.
	2nd day.	++++	0.4 per cent.	10	50	44	
	3rd "	+++	+	10	25	25	
	4th "	++	+	5	50	30	Rabbit found dead next morning.
1,535	1st day.	+++	Trace.	35	200	200	21 cc. M/8 $CaCl_2$ followed by 21 mg. zinc as malate injected intravenously.
	2nd "	++++	0.5 per cent.	35	170	12	Rabbit lived 12 days. Traces of albumin found after 5th day.
	3rd "	+++	+	5	60	75	Appearance of sugar irregular after 2nd day.
	4th "			20	60	None.	
	5th "	+	None.	5	90	55	
1,508	1st day.	Trace.	None.	5	50	80	15 mg. zinc as malate injected intravenously and followed by 15 cc. M/8 $CaCl_2$.
	2nd and 3rd days.	++	0.2 per cent.	None.	100	34	
	4th day.	+++	+	"	40	80	Found dead next morning 9.00 a.m.
1,515	1st day.	++	None.				13 mg. zinc malate injected intravenously followed by 13 cc. M/8 $CaCl_2$. Urine examined daily or every other day for 15 days after injection of zinc and calcium. No sugar found.
	2nd and 3rd days.	Trace.	"	55	150	65	

well as albuminuria, was frequently absent in carrot-fed rabbits. When calcium chloride was injected intravenously immediately after or before zinc malate, glycosuria was slightly increased. The rate of secretion of urine was either diminished or remained unchanged after the administration of zinc salts. In a few cases only was the amount of urine augmented after the zinc.

It appears, therefore, from this summary that some of the effects produced by zinc salts are analogous to those caused by the administration of other heavy metals. On the other hand, well marked differences were also observed. While hyperglycemia, after the subcutaneous injection of potassium chromate, was reported by Pollak,[15] this was denied by Kóssa,[17] Frank,[5] and later by Luzzatto.[6] This writer observed a slight hyperglycemia after mercuric chloride, but it was not corroborated by other investigators.

The duration of zinc glycosuria also seemed to be different from that observed after other heavy metals. As reported by Frank,[5] glycosuria persisted in dogs 8 to 11 days after the subcutaneous administration of uranium nitrate. He claimed to have observed a similar tendency in experiments with potassium chromate, but his protocol shows that the glycosuria lasted 3 to 4 days only. Considerable emphasis was laid by Luzzatto and by Frank on the polyuria observed in the early stages of poisoning with large doses and of its persistence when small doses were given. The protocols cited and a large number of experiments made with zinc in another connection indicated that the volume of urine may, on the contrary, be decreased at the beginning or remain unchanged, polyuria occurring in some cases only. According to some observers the appearance of sugar preceded the albuminuria, but others have reported that the order was reversed. With the exception of the hyperglycemia the character of the glycosuria after zinc malate is closely similar to that produced by the other heavy metals. It has been pointed out by several observers that the glycosuria caused by heavy metals is due to increased permeability of the kidney for sugar and has been regarded, therefore, as renal diabetes. The mechanism of the glycosuria produced by zinc salts is even more difficult of determination since, as we have seen, it deviates somewhat from the type produced by other heavy metals. Several factors may be considered

before drawing conclusions as to the nature of zinc glycosuria. The depression, and finally the paralysis, of the respiratory center produced by zinc salts was long ago pointed out by Harnack.[27] This has been corroborated by numerous experiments upon rabbits carried out in this laboratory with zinc malate. That diminished respiration and asphyxia cause glycosuria was shown by several investigators. Araki[28] reported the presence of large amounts of sugar in the urine of different animals that have been kept in an atmosphere poor in oxygen. Kunkel[29] stated that 6 per cent of sugar was found in the urine of animals in asphyxia. Later Underhill[30] found that lack of oxygen may produce a marked hyperglycemia as well as glycosuria. According to Araki glycosuria following asphyxia is possible only in well fed animals. Hence the glycosuria produced by zinc malate is in all probability not due to diminished respiration and consequent decrease of oxidation, since glycosuria caused by zinc may be produced in fasting, and the amounts of sugar present in the urine are very small. There is reason to believe that renal diabetes, which may be due to changes in the glomeruli or to injury of the convoluted tubules, is inhibited by calcium. Our findings with this salt, as stated above, might indicate that the glycosuria is not of renal origin, since the administration of calcium salt in zinc diabetes gave negative results. On the other hand, we found that rabbits behave differently when they are fed oats alone, or when they receive carrots exclusively. This is, indeed, significant, in view of the protective action of diet against renal irritants, reported by Salant and Bengis,[31] and Salant and Swanson.[32] The absence of sugar in the urine of rabbits that received zinc, notwithstanding a diet very rich in sugar, might indeed be due to the increased resistance of the kidney to this metal, which furnishes a strong argument in favor of the renal origin of zinc glycosuria.

[27] Harnack, E., *Arch. exp. Path. u. Pharmakol.*, 1874–75, iii, 44.

[28] Araki, T., *Z. physiol. Chem.*, 1891, xv, 335.

[29] Kunkel, A. J., Lehrb. Toxikol., Jena, 1901, pt. i, 348.

[30] Underhill, F. P., *J. Biol. Chem.*, 1905–06, i, 113.

[31] Salant, W., and Bengis, R., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 529.

[32] Salant, W., and Swanson, A. M., *J. Pharmacol. and Exp. Therap.*, 1918, xi, 27.

THE DISTRIBUTION AND ELIMINATION OF ZINC AND TIN IN THE BODY.

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The presence of zinc in the tissues and fluids of the body after the administration of its compounds to animals was studied by Heller,[1] Michaelis,[2] Schlossberger,[3] Matskevitch,[4] Lehmann,[5] Sacher,[6] Jacobi,[7] and Brandl and Scherpe.[8] The different results obtained by these observers may be represented by the work of the following investigations. After the intravenous injection into a dog, of 300 mg. of zinc in the form of malate, which was introduced at different intervals in 7 days, Jacobi recovered from the feces 70.9 per cent in 14 days. 7 days after the injection of zinc was discontinued, the dog was bled and the organs were removed from the body. Analysis for the presence of the metal indicated that the liver contained 8.4 per cent of the amount introduced in the circulation, but mg. doses only were found in the spleen and kidneys. According to Matskevitch zinc oxide or acetate injected subcutaneously into dogs is eliminated chiefly by the stomach and intestines, smaller quantities being found in the urine and in the bile, but he reported that 35 per cent of the quantity introduced was found in the skeleton, and 4 per cent in the skin. Lehmann stated that after feeding zinc carbonate to a dog for 11 months he found zinc in considerable quantities in the liver, kidney, lungs, urine, bile, stomach, and intestines.

The present report deals with the excretion of zinc when administered by various methods, but experiments were also conducted to ascertain how long it remained in the circulation, and whether it was deposited in any of the organs. Additional

[1] Heller, J. F., *Arch. Phys. u. path. Chem. u. Mik.*, 1847, iv, 231.
[2] Michaelis, F., *Arch. physiol. Heilk.*, 1851, x, 109.
[3] Schlossberger, *Arch. physiol. Heilk.*, 1848, vii, 589.
[4] Matskevitch, B., Dissertation, St. Petersburg, 1878.
[5] Lehmann, K. B., *Arch. Hyg.*, 1897, xxviii, 291; 1910, lxxii, 358.
[6] Sacher, A., *Arb. pharmakol. Inst. Dorpat*, 1893, ix, 88.
[7] Jacobi, *Arb. k. Gesundhtsamte*, 1899, xv, 204.
[8] Brandl and Scherpe, *Arb. k. Gesundhtsamte*, 1899, xv, 185.

data on the elimination and distribution of tin are also included in this investigation in continuation of the work on the same subject previously published from this laboratory.[9]

Method.

Zinc malate and acetate were administered by mouth, subcutaneously, and intravenously. The elimination of zinc into the gastrointestinal canal was studied by examining the contents of the stomach and intestines, and the feces, separately or combined. The urine, blood, bone, liver, and skin were also analyzed for zinc. The method of analysis was as follows: The material was digested with nitric and sulfuric acid until a clear and almost colorless solution was obtained. The acid was then evaporated and the residue ignited in a muffle at a low red heat and extracted with about 5 per cent hydrochloric acid several times. A few cc. of a 10 per cent solution of sodium dihydrogen phosphate and enough ammonia to make it strongly alkaline were then added to the extract, which was about 0.5 to 1 liter. This was filtered and the precipitate washed and discarded. The filtrate and washings, which contained the zinc compound in solution, were evaporated to dryness. When the residue was small, it was dissolved in water containing a few cc. of ammonia with 1 or 2 cc. of the acid phosphate, and the solution almost exactly neutralized (alizarin). Under these conditions zinc ammonium phosphate is formed and upon shaking for a few minutes settles out as a flocculent precipitate which becomes granular on standing for several hours in the cold. It is then filtered off on a tared Gooch crucible, ignited at a low red heat, and weighed as zinc pyrophosphate. To confirm the presence of zinc, the zinc pyrophosphate should be dissolved from the Gooch and identified as the white sulfide. In cases where the residue obtained by evaporation was considerable, it was ignited again after adding a few cc. of concentrated sulfuric acid, and the process carried on from that point in the usual way.

EXPERIMENTAL.

The fate of zinc when injected intravenously indicated that the gastrointestinal canal was the chief organ of elimination, as 2 or 3 days after its administration one-third to nearly one-half of the amount given was recovered from its contents and the feces. The urine, as shown in Table I, contained but minute quantities of zinc which were practically within the limits of experimental error. Appreciable amounts of the metal were recovered from the liver. In three experiments, it was 13 to 15 per

[9] Salant, W., Rieger, J. B., and Treuthardt, E. L. P., *J. Biol. Chem.*, 1914, xvii, 265.

cent; in the fourth it amounted to about 11 per cent of the quantity of zinc administered. Although the amounts present in the skin were small, 2.7 and 4.2 mg. of zinc, the evidence is sufficient to warrant the conclusion that the metal may either be stored or eliminated by this organ. An attempt was also made to study the elimination of zinc in abnormal conditions of the intestine. According to McCrudden,[10] elimination by the intestinal mucosa takes place more readily if hyperemia is present. Concentrated solutions of sucrose were given by mouth to rabbits as this produces congestion and sometimes inflammation of the mucous membranes of the intestine. The analytical data obtained indicated, on the contrary, that much smaller quantities of zinc are eliminated by the intestines after this treatment. The amounts recovered in three experiments were 22.5, 20, and 11 per cent of the quantities injected intravenously, which practically represented the elimination by the gastrointestinal canal as the quantities found in the liver, when sucrose was fed, were negligible, being 0.9 mg. of zinc in one experiment, 1.2 mg. in another, and 2.5 mg. in a third.

Observations on the fate of zinc when injected intravenously were also made on cats into which zinc malate was administered under local anesthesia. Three cats used for these experiments received a total of 84 mg. of zinc in the form of malate, and were bled 3 hours later. The results of the analysis are as follows:

Gall bladder and contents	Less than 1 mg. Zn.
Liver	2.8 " "
Brain	None.
Skin	6 mg. Zn.
Kidneys, spleen, and heart	Trace.
Intestines and contents	8.4 mg. Zn.
Stomach and contents	Trace.
Muscle (considerable loss)	4.4 mg. Zn.
Blood	8.4 " "
Bones (only small part of skeleton examined, rest lost)	2.4 " "

It will be observed that even after an interval of only 3 hours, the elimination into the intestines was about 10 per cent of the amount injected but excretion into the stomach or the bile may

[10] McCrudden, F. H., *Arch. exp. Path. u. Pharmakol.*, 1910, lxii, 374.

TABLE I.

Elimination and Distribution of Zinc in Rabbits.

Experiment No.	Urine.	Feces.	Contents of gastrointestinal canal.	Liver.	Blood.	Skin.	Bones.	Zinc injected intravenously.	Total zinc recovered.	Elimination by gastrointestinal canal.	Remarks.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	
VIII	1.0	3.0	14.2	4.7	No examination.	No examination.	No examination.	36.0	63.9	47.7	
IX	2.0	18.5	1.5	7.6	" "	" "	" "	48.0	61.66	41.7	Three rabbits used. Chloroformed 72 hrs. after injection of zinc. Diet, oats.
X	1.5	13.3	14.6	9.9	1.3	2.7	" "	63.3	69.5	44.4	Diet, oats. Three rabbits used. Bled 2 days after injection of zinc malate and blood examined for zinc.
XI	1.7	21.1	None.	7.2	No examination.	4.2	" "	63.3	54.5	33.3	Diet, oats. Three rabbits chloroformed 2 days after injection of zinc malate.

be considered negligible. The intestines may be regarded, therefore, as the only channel for the elimination of zinc also in this case. The small amount of zinc in the liver is probably related to its presence in the circulation, accumulation in this organ taking place upon the disappearance of the metal from the blood.

The elimination of zinc when given by subcutaneous injection likewise took place chiefly in the intestinal canal, but much smaller quantities of the metal were found in the contents of the intestines and feces in these experiments than after intravenous injections. The amounts of zinc recovered in four experiments were 10, 15, 30, and 32.2 per cent. This would seem to be accounted for by the retarded absorption as indicated by the larger amounts of zinc found in the skin at the site of injection, but the total amount of zinc in the skin was 67 mg. The remainder, which is 114 mg. of the metal, must have been absorbed. Similar results were obtained in another experiment. As in experiments in which zinc was given intravenously, the amounts eliminated in the urine in 48 hours were small, being 1.0, 2.0, and 2.5 mg. Only small amounts were found in the liver. Analytical data of rabbits that received zinc by subcutaneous injection follow:

Total Zn Injected, 181 Mg. (88 and 93 Mg.).

	mg.	
Combined urines	2.5	
Feces, Rabbit 1,103	4.3	10 per cent
Gastrointestinal canal, Rabbit 1,103	5.2	
Feces, Rabbit 1,104	6.0	15 per cent
Gastrointestinal canal, Rabbit 1,104	8.0	
Combined livers and kidneys	5.5	
Skin, Rabbit 1,103, region of injection	27.0	
" " 1,103, remainder	8.5	
" " 1,104, region of injection	29.5	
" " 1,104, remainder	11.0	
Total amount of zinc recovered in 48 hrs.	107.5 = 59.4 per cent of amount injected.	

Experiments were also carried out to test the absorption of zinc from the gastrointestinal canal. 1 gm. of zinc acetate was given by mouth through a stomach tube to each of three rabbits. At the end of 24 hours, the rabbits were bled to death. The

blood of the three rabbits was combined and examined for zinc, but traces only could be detected. The livers which were similarly treated, contained 7.0 mg. of zinc. In a second experiment in which the zinc acetate was given 24 hours after feeding 50 cc. of a 60 per cent solution of sucrose, the amount of zinc in the combined livers was 12.5 mg. In two other experiments with zinc acetate in which no sucrose was given, the liver contained 3.3 and 4.0 mg. of zinc. It would seem, therefore, that the sugar probably favors the accumulation of zinc in the liver. This may be due to increased absorption as the strong solution of sugar was usually followed by well marked hyperemia and often by inflammation of the intestine. It has been shown by Mayerhofer and Přibram[11] that absorption from the intestine is more favorable in animals in which enteritis has been produced experimentally. Perhaps the same holds for zinc.

DISCUSSION.

The evidence regarding the fate of zinc in the body indicates that the distribution in the tissues and the channels of elimination are closely similar for all heavy metals. As has been shown for lead, silver, iron, nickel, mercury, and bismuth, the gastro-intestinal canal is the main channel of elimination, the kidney playing a very subordinate rôle. In the case of some of these substances, the result of analysis of the urine either failed to indicate their presence after their administration to animals or mg. doses only were found as has been shown in the case of lead. According to Legge and Goadby,[12] the amounts eliminated by the kidney never exceeded 5 mg. in 24 hours, while Blum,[13] reported that he was unable to detect the presence of lead in the urine after poisoning with lead iodide, but the kidneys contained minimal amounts of it. This is particularly interesting in view of the fact that the renal changes produced by these metals are strikingly similar, according to Legge and Goadby. The kidney is likewise apparently unable to eliminate either iron or silver.

[11] Mayerhofer, E., and Přibram, E., *Z. exp. Path. u. Therap.*, 1909–10, vii, 247.

[12] Legge, T. M., and Goadby, K. W., Lead Poisoning and Lead Absorption, London, 1912.

[13] Blum, F., *Wien. med. Woch.*, 1904, liv, 537.

TABLE II.

Elimination and Distribution of Tin after Intravenous Injection.

Cat No.	Urine.	Intestine and contents.	Stomach contents.	Stomach wall.	Liver.	Spleen.	Lungs.	Brain.	Muscle.	Skin.	Skeleton.	Blood.	Heart.	Kidneys.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
97	Not examined.	8.0	0.6	2.3	7.1	1.7	2.7	0.4	22.3	11.0	36.8	Not examined.	Not examined.	—	Ether anesthesia; injected 122.9 mg. tin intravenously. Died 1¾ hrs. after injection.
182	7.6	.6		1.6	8.4	0.7	4.1	0.4	9.0	Not examined.	5.9	7.1	0.7	5.0	Ether anesthesia; injected 110 mg. tin intravenously. Bled 2 hrs. after injection.